ASSESSING DISEASE CONCERNS ON QUINOA AND EVALUATING SOURCES OF DISEASE RESISTANCE IN *CHENOPODIUM* SPECIES IN NEW ENGLAND

BY

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B.S. Biology, University of Central Florida, 2017

THESIS

Submitted to the University of New Hampshire

in Partial Fulfillment of

the Requirements for the Degree of

Master of Science

in

Genetics

September 2019

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On June 18th, 2019

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ABSTRACT

ASSESSING DISEASE CONCERNS ON QUINOA AND EVALUATING DISEASE RESISTANCE IN *CHENOPODIUM* SPECIES NATIVE TO NEW ENGLAND

By

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Quinoa (*Chenopodium quinoa* Willd.) is a potential new crop for New England because of its high nutritional qualities and environmental adaptability; however, its susceptibility to disease such as downy mildew, caused by *Peronospora variabilis* Gaum is a significant obstacle. Downy mildew is a key disease of quinoa causing yield reductions up to 33% in tolerant varieties and 99% yield loss in susceptible varieties. Warm temperatures and high humidity in New England provide optimum conditions for pathogen infection and spread, negatively impacting quinoa growth and yield. This pathogen is able to persist in the seed coat of quinoa and overwinter in soils, making it difficult to manage in a farm setting. These characteristics, as well as reports of fungicide resistance in other downy mildew systems, make genetic resistance the most effective strategy to combat this pathogen. *P. variabilis* has been reported in North America, specifically in Alberta, Canada and Pennsylvania however it is not known to occur in New England. This research serves as preliminary work for future studies to identify genes contributing to disease resistance in New England native *Chenopodium* species for future use in breeding programs. The specific objectives of this research were to (1) optimize molecular protocols to detect *P. variabilis* and confirm the presence of the pathogen in New Hampshire, (2)

evaluate differential resistance to downy mildew among *Chenopodium* accessions, (3) identify other potential pathogens of *Chenopodium* spp. in New England, and (4) investigate molecular relationships among New England *P. variabilis* isolates. A molecular pipeline involving touchdown PCR was developed and used to confirm the presence of *P. variabilis* on *Chenopodium* species in New Hampshire. Field trials conducted at the UNH Woodman Research Farm evaluated downy mildew disease severity on ten *Chenopodium* accessions representing four species. Disease severity for each treatment was compared and significant differences were observed among treatments. *C. berlandieri* var. *macrocalycium* ecotypes collected from Rye Beach, NH and Appledore Island, ME exhibited the lowest mean disease severity over the season. *P. variabilis* was isolated from each of the 10 accessions and COX2 sequences were compared; phylogenetic analyses suggest no effect of host species; however, New Hampshire isolates formed a clear cluster when compared with Pennsylvania and South American isolates, suggesting the presence of distinct populations based on geographic location. A second disease causing stem lesion symptoms was observed in the 2018 field trial. Stem lesion incidence was significantly different among *Chenopodium* accessions, with certain quinoa accessions (37P, QQ065, cultivar Faro) exhibiting low incidence; however, definitive conclusions were not reached in regard to the identity of the stem lesion causal agent. Overall, results from this study provide the first step in identification of potential New England native sources of resistance to downy mildew within the genus, confirmation that downy mildew is present and may be problematic for New England quinoa growers, and documentation of another unknown quinoa pathogen that will need to be managed. These results also provide preliminary information needed to further investigate resistance at the genomic level in *Chenopodium* spp. and pathogenicity factors in *P. variabilis*.

CHAPTER ONE: INTRODUCTION

Quinoa, *Chenopodium quinoa* Willd., is a highly nutritious crop native to the Andean region in South America where it has been cultivated for over 7,000 years (Danielsen and Ames, 2004). Due to its high nutritive properties and adaptability to harsh environments, quinoa is a promising, potential new crop for North American agriculture (Testen et al., 2014). Quinoa has recently gained worldwide attention because of its desirable agronomic characteristics including the ability to grow in high stress conditions such as high soil salinity, high soil acidity, drought, and frost (Kumar et al., 2006). This pseudocereal is also gaining attention for its high-quality protein content, essential fatty acid content, natural antioxidants, and its wide range of vitamins and mineral content (Kumar et al., 2006). Despite its many useful traits, there has been little genetic improvement of quinoa related to its agronomic performance. For example, excessive branching, lodging, susceptibility to disease, and sensitivity to high temperatures have placed constraints on commercial production of the crop (Maughan et al., 2018). Recently, Dr. Tom Davis at the University of New Hampshire began investigating the potential to grow quinoa as a specialty crop in New England. The Davis Lab is working on a multi-year project to "re-domesticate" quinoa in New England. In this project, researchers are investigating the use of wild/weedy locally adapted *Chenopodium* species as a breeding partner with the Andean quinoa to develop cultivars suitable for crop production in New England.

Downy mildew, caused by *Peronospora variabilis* Gaum is one of the most economically important diseases of quinoa, reducing yield up to 33% in tolerant varieties and 99% in susceptible varieties (Alandia et al., 1979; Bhargava et al., 2006; Testen et al., 2014). *P.*

variabilis was initially reported as endemic in South America; however, the pathogen has been reported causing disease on *C. quinoa* in Canada in 1990 and more recently in the United States in 2012 (Testen et al., 2014, Tewari and Boyetchko, 1990). This finding is especially noteworthy as there is limited knowledge of the pathogen distribution in North America.

Obstacles for North American quinoa cultivation

While disease is a significant obstacle in achieving efficient quinoa cultivation in North America, there are many other physical characteristics of quinoa that prevent proper growth and harvesting such as excessive branching, lodging (bending over of plants), shattering of seeds, and sensitivity to humidity (personal observations; Maughan et al., 2019). Additionally, quinoa is a crop that is native to and domesticated in high altitude regions of the Andes, a region with a much longer growing season than that in New England. Quinoa varieties are adapted to this longer growing season and when grown in northern regions, seed heads fail to mature before fall frosts begin. These challenges, as well as the presence of *P. variabilis* in North America, present a significant obstacle to redomesticating quinoa and integrating the crop into North American agriculture.

Agronomically important diseases of quinoa

While downy mildew is the most damaging and widespread disease of quinoa, several other diseases have been reported around the world and are known to significantly reduce quinoa growth and yield (Danielson et al., 2003).

Leaf and stalk diseases

There are two prevalent leaf and stalk diseases; quinoa leaf spot and brown stalk rot, caused by *Ascochyta hyalospora* (Cooke and Ellis) and *Phoma exigua* var. *foveata* (Foister) Boerema, respectively (Danielsen et al., 2003). *A. hyalospora* was reported on quinoa in the

United States in 2011 in Centre County, Pennsylvania (Testen et al., 2013). Symptoms begin as reddish-brown foliar lesions which ultimately develop into circular necrotic spots with black asexual fruiting bodies, known as pycnidia, distributed within the lesions (Testen et al., 2013). The impact of *Ascochyta* leaf spot has on quinoa production is unknown (Testen et al., 2013), but management of foliar diseases is integral for disease management and overall crop health.

Phoma exigua var. *foveata* has been reported in the Netherlands (Irinyi et al., 2009) and frequently in the Andes (Alandia et al., 1979). The infection begins as small lesions on the upper third of the stalk. Lesions expand to cover more area of the stalk and inflorescence (Danielsen et al., 2003). A diagnostic sign of this disease is presence of pycnidia, and key symptoms include chlorotic leaves and downward bending stalks that break easily (Danielsen et al., 2003). *P. exigua* var. *foveata* is a soil borne pathogen favored by low temperatures and high humidity and is also known to infect other crops such as potato (Danielsen et al., 2003). It has also been reported that *P. exigua* var. *foveata* requires natural openings (created by mechanical injury) for host invasion and infection, leading to frequent outbreaks in the Andes after hailstorms (Danielsen et al., 2003).

Root rot and damping-off diseases

The number of root and seedling diseases of quinoa is difficult to discern because observations of some root and seedling diseases are not always officially reported in the USDA National Fungal Database (Farr and Rossman, 2019), or reported in the scientific literature. Researchers at the International Potato Center in Lima, Peru isolated *Rhizoctonia solani* and *Fusarium* species from quinoa in 1998 (Barboza et al., 2000). It was found that *R. solani* produced damping off symptoms such as failure of seed germination and sunken stem lesions (Danielsen et al., 2003). The *Fusarium* spp. isolated were reported to cause wilting and root rot

(Danielsen et al., 2003). Seed rot and damping off has also been seen to be caused by *Sclertoium rolfsii* Sacc. and *Pythium zingiberum* (Danielsen et al., 2003). *S. rolfsii* was first reported on quinoa in California in 1980 (Beckman and Finch 1980). Disease symptoms of *S. rolfsii* infection include stem girdling and plant collapse (Danielsen et al., 2003). *P. zingiberum* also causes rhizome rot in ginger and its oospores have been reported to cause damping off in quinoa seedlings (Iked and Ichitani, 1985, Danielsen et al., 2003).

There are still many unknown factors of the pathology and epidemiology of quinoa pathogens. Even for downy mildew, the plant's most prevalent disease, information such as epidemiology, host specialization, population structure, and host plant resistance are largely unknown, and this knowledge gap is applicable to other diseases as well (Danielsen et al., 2003).

Quinoa downy mildew

Symptoms and signs

Symptoms are a plant's response to a pathogen, and generally, signs are the presence of the actual pathogen itself. *P. variabilis* causes symptoms of leaf chlorosis in all host species and may also cause pink discoloration of leaves on a few *Chenopodium* species (Testen et al., 2012). The disease has also been reported to drastically reduce quinoa yield: stunting growth rate and yield anywhere from 33% in tolerant varieties to 99% in highly susceptible varieties (Testen et al., 2014). A diagnostic sign of this pathogen is gray to black fuzzy asexual sporulation on the underside of the leaf surface. *P. variabilis* grows within the intercellular space of leaf tissue and blocks photosynthetic activity, resulting in the development of necrotic spots on the leaves and eventual defoliation.

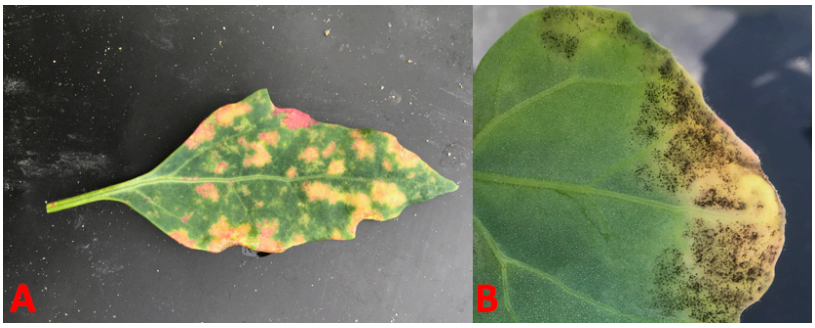

Figure 1-1. Symptoms (A) and sign (B) of downy mildew on *Chenopodium quinoa* x *C. berlandieri* var. *macrocalycium* hybrid during Summer 2018 field trial. Photo: H. Nolen.

Distribution

P. variabilis has been documented throughout the world wherever quinoa is cultivated. The pathogen has been reported on the quinoa weedy relative *Chenopodium album* (lambsquarters) in Argentina (Choi et al., 2010), Central Asia (Gaponenko, 1972), China (Choi et al., 2008, 2010), Germany (Riethmuller et al., 2002, Goker et al., 2007, Choi et al., 2008, 2010), Ireland (Choi et al., 2008, 2010), Italy (Choi et al., 2008, 2010), Korea (Choi et al., 2008, 2010), Latvia (Choi et al., 2008, 2010), Netherlands (Choi et al., 2008, 2010), Pakistan (Ahmad et al., 1997), Poland (Kucmierz, 1966), Romania (Choi et al., 2008, 2010), and Switzerland (Constantinescu, 1991); on *Chenopodium murale* in India (Baiswar et al., 2010); on *Chenopodium quinoa* in Korea (Choi et al., 2014), Canada (Tewari and Boyetchko, 1990), and the United States (Testen et al., 2012); and on an unidentified *Chenopodium* species in Ireland (Muskett and Malone, 1984).

P. variabilis systematics and diagnostic structures

The species *Peronospora variabilis* Gaum (formerly *P. farinosa* f. sp. *chenopodii* Byford) is a part of a larger group of organisms classified as downy mildews. All downy mildew diseases are caused by oomycete fungal-like organisms within the Peronosporaceae family, class Oomycota, phylum Stramenopiles, and Kingdom Protista. Members of this phylum are largely

obligate parasites, otherwise known as biotrophs, that cannot be cultured on artificial media outside of their host.

While oomycetes are not classified as true fungi, they behave and grow very similarly to fungi. Oomycetes are considered distinct from true fungi because they do not have a cell wall composed of chitin, but cellulose and glycan (Sleigh, 1990). Another main difference between oomycetes and true fungi is that oomycete filaments contain diploid nuclei, whereas fungal filaments contain haploid nuclei (Sleigh, 1990). *P. variabilis* is an organism with vegetative and reproductive morphological stages within its life cycle. The pathogen's vegetative structures are hyphae that produce fruiting bodies (sporangiophores, 2n) bearing asexually produced spores (sporangia, 2n). Sporangiophores are tree-shaped structures that are dichotomously branched 4-5 times at acute angles and have 2-3 serpentine ends in which sporangia are attached (Figure 1-2, Danielsen and Ames, 2004). Sporangia detach from the sporangiophore once they have reached maturity (Danielsen and Ames, 2004). These sporangia are able to germinate directly without producing zoospores (unlike many other oomycete pathogens). Oospores (2n) are the organism's sexual spores that can lay dormant and overwinter within the soil between growing seasons and can be found in the pericarp of quinoa seed (Testen et al., 2014; Danielsen et al., 2004). Oospores of *P. variabilis* are light brown/orange rounded, double-walled structures. Oospores are produced from the fusion of male gametangia (antheridia) with female gametangia (oogonia) and the fusion of their haploid nuclei. *P. variabilis* is heterothallic, meaning that the two gametangia must come from different thalli. In *P. variabilis*, two mating types, P1 and P2, have been reported in Peru and Bolivia (Danielsen et al., 2000; Danielsen and Ames, 2004). Sexual

reproduction provides the opportunity for *P. variabilis* to undergo recombination and affect the genetic variation of the species.

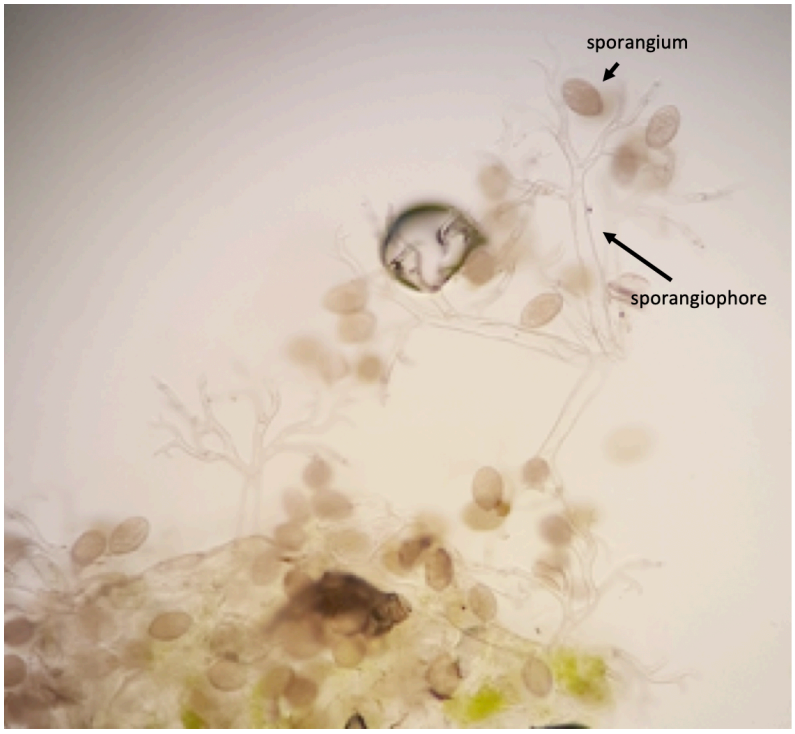

Figure 1-2. Microscopic images at the 40X objective of *P. variabilis* sporangia and sporangiophores isolated from a *C. album* plant in Durham, NH. Photo: A. Poleatewich.

Life cycle

The asexual infection cycle for *P. variabilis* starts with a sporangium landing on the leaf of a susceptible plant. Free moisture on the leaves is necessary for the sporangium to germinate and penetrate the plant epidermis with mechanical force. Upon penetration, hyphae spread intercellularly. Approximately 5-6 days after penetration occurs, sporangiophores develop and protrude through the stomata on the underside of the leaf (Danielsen and Ames, 2004). These sporangiophores produce asexual sporangia, initiating the secondary cycle of the disease. Downy mildew caused by *P. variabilis* is characterized as a polycyclic disease meaning that several infection cycles occurring within one growing season (Danielsen and Ames, 2004). The number

of cycles in a season will depend on environmental conditions, specifically temperature and leaf wetness.

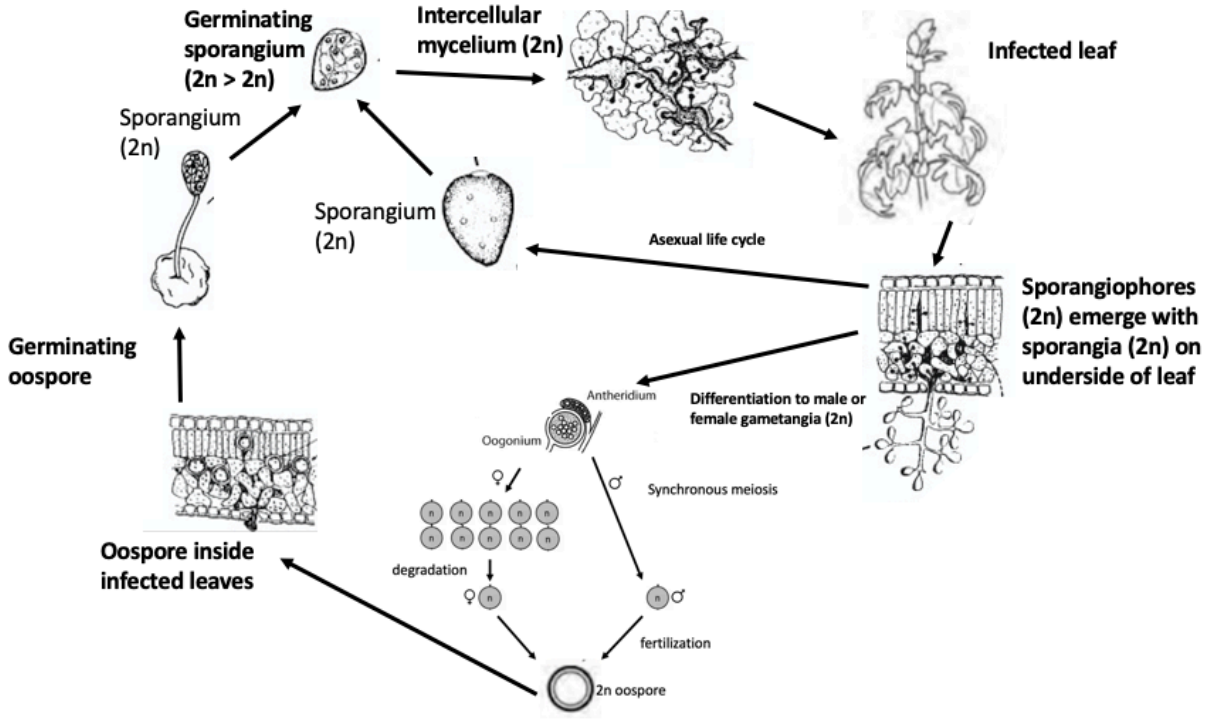

Figure 1-3. *P. variabilis* life cycle, adapted from Lizotte (2015), Ellis (2016), Jackson (2008), Kandel et al. (2019), and Danielsen and Ames (2004).

Downy mildew taxonomy

The Oomycota class in the Stramenopiles phylum contains many economically important plant pathogens, such as *Pythium* spp., *Phytophthora* spp., and downy mildews. Organisms within Oomycota are believed to have stemmed from biflagellate, free-ranging predatory protists, with marine basal lineages (Thines and Choi, 2016). An evolutionary transition to a terrestrial lifestyle is thought to have been made at least four times within the class (Thines and Choi, 2016). A key diagnostic feature of the class is the presence of sexual oospores (Thines and

Choi, 2016). This class contains a crown group, the Peronosporomycetes, containing the many downy mildew causal organisms, collectively called the downy mildews (Thines and Choi, 2016). Downy mildews make up more than a third of known oomycete species and were split into three major monophyletic groups by Dick (1995): downy mildews with colored conidia (DMCC) with approximately 500 species; downy mildews with pyriform haustoria (DMPH) with 150 species; and Brassicolous downy mildews (BDM) with 100 species (Figure 1-4, Thines and Choi, 2016). Recently however, it has been seen that two of these downy mildew groups (GDM and BDM) are not monophyletic, and maximum likelihood trees including downy mildews and *Phytophthora* species suggest the possibility that the BDM group arose from a host jump from a graminicolous ancestor (Figure 1-5, Bourret et al., 2018).

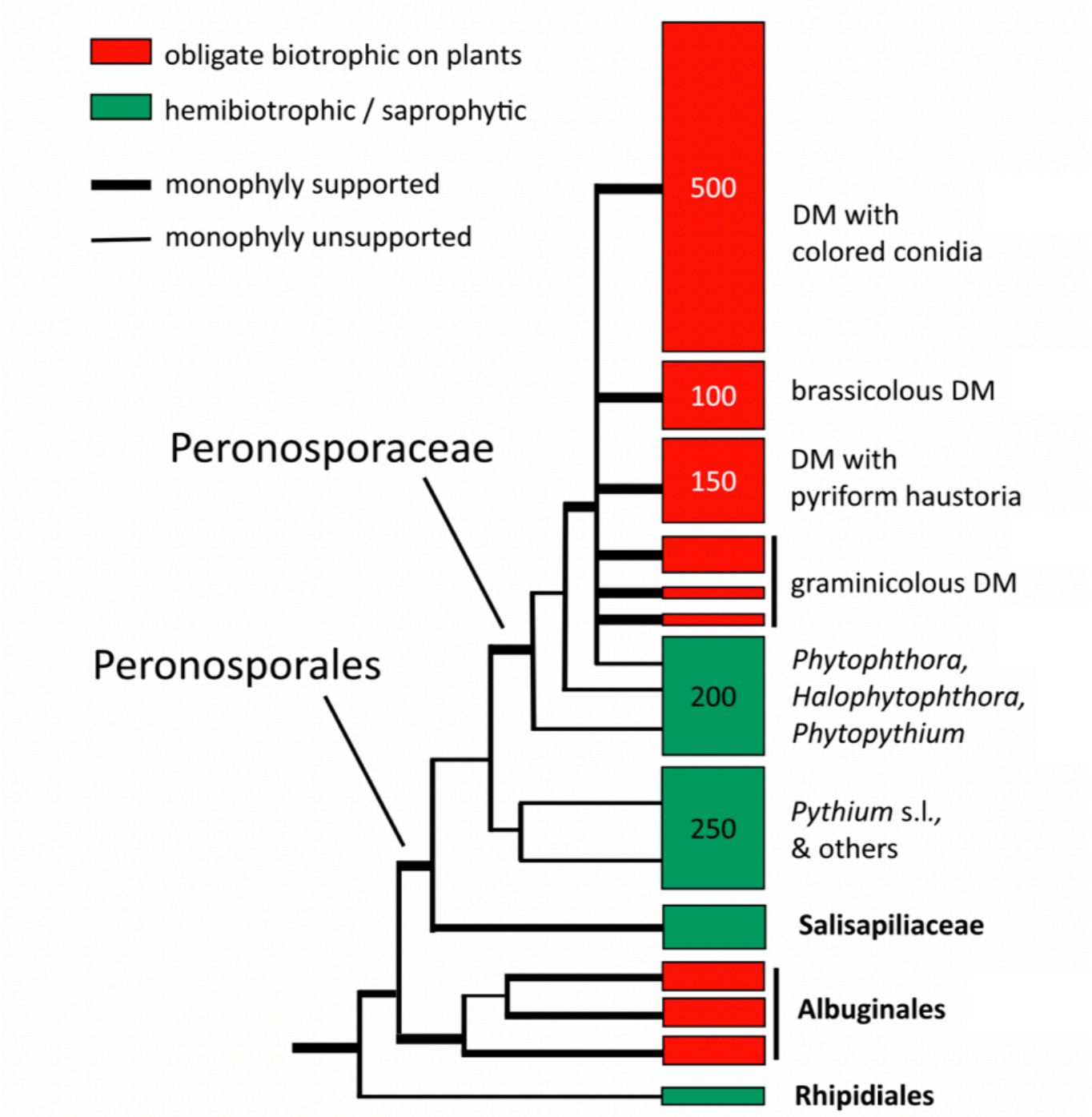

Figure 1-4. Schematic overview of the Peronosporomycete phylogeny by Thines and Choi (2015). Numbers in boxes are approximate species numbers.

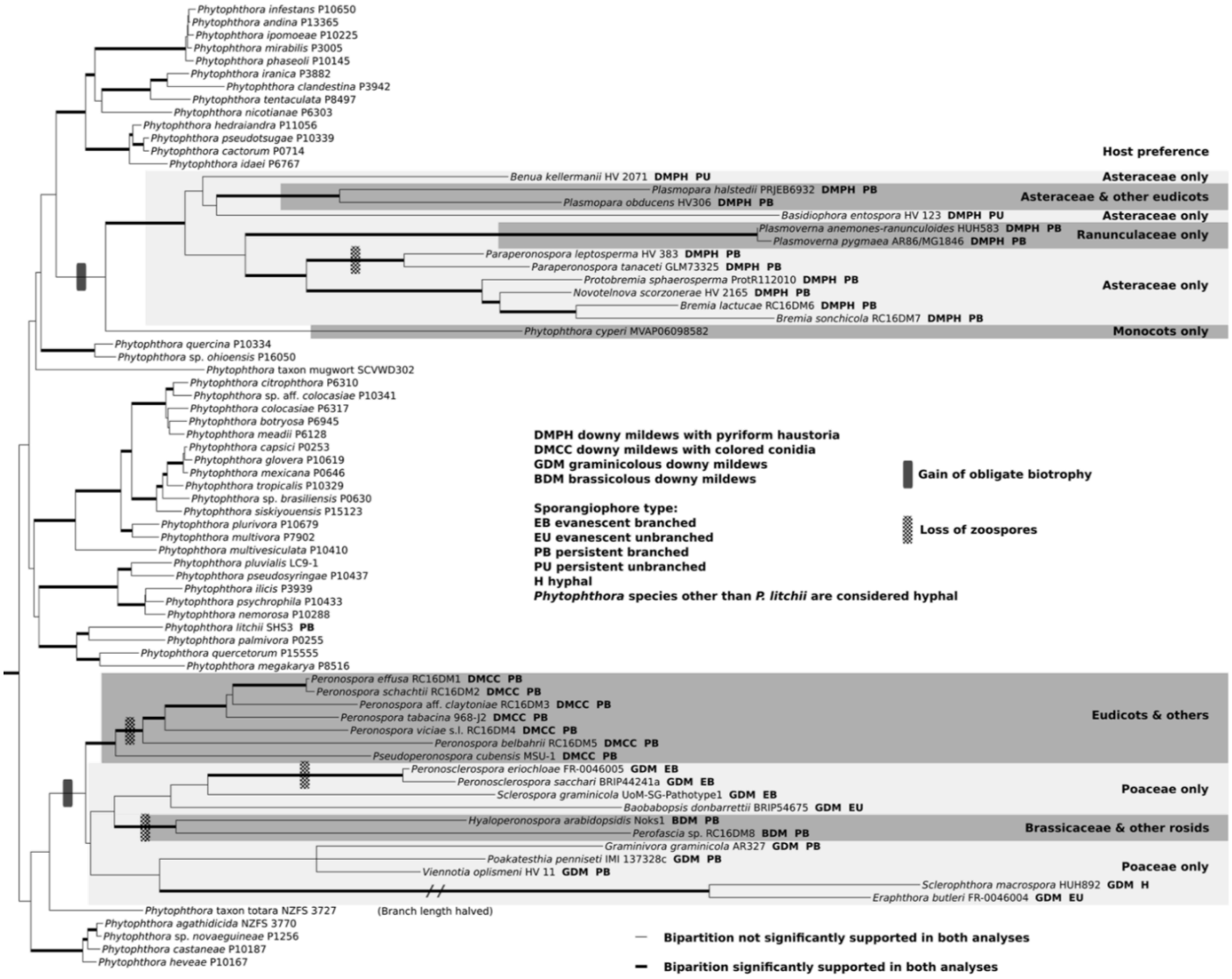

Figure 1-5. Maximum likelihood tree inferred by Bourret et al. (2018)

The genera *Peronospora* and *Pseudoperonospora* fall into the DMCC group (Figure 1-4). A diagnostic feature of the organisms in this group is the presence of melanized sporangia, the evolution of which is thought to be a key factor in the evolutionary success of the group, allowing longer survival under radiation exposure and during wind transport over long distances (Thines and Choi, 2016). An important trait shared by all downy mildews as well as members of

the order Albuginales is obligate biotrophy, which requires the organisms to feed off of a living plant host to survive (Thines and Choi, 2016). This characteristic seems to have evolved independently in these two lineages of the Peronosporomycetes, with all other lineages being hemibiotrophic (surviving on living or dead tissue) or saprophytic (surviving on only dead tissue) (Figure 1-4) (Thines and Choi, 2016). While there are many economically significant plant pathogens within the downy mildew and oomycete groups, many aspects of their biology and evolution are still unknown (Danielsen et al., 2003; Thines and Choi, 2016).

Hypotheses of differential strains (special forms) of P. variabilis

An important characteristic of downy mildew diseases that has piqued the interest of many researchers is their host specificity. Most downy mildews are host-genus or even host-species specific, meaning a certain downy mildew pathogen will only infect and cause symptoms on certain members of a host genus or species (Thines et al., 2009). This hypothesis is backed by the broad species concept justified by de Bary (1863) and Yerkes and Shaw (1959). This concept attributes the host specificity of some downy mildew pathogens to the presence of genetically distinct specialized forms of the same species (Thines and Choi, 2016). Several studies have provided evidence of a high-degree of specialization within *Peronospora* (Thines and Choi, 2016; Belbahri et al., 2005; Choi et al., 2008, 2010). It seems that an evolutionary phenomenon known as host jumping is likely to have been an important factor in the evolution of downy mildews and the *Peronospora* genus. This hypothesis is due to host and pathogen phylogenies not being congruent within pathogen groups restricted to a specific host family, as these phylogenies would be expected to be congruent if the host and pathogen continually co-evolved (Thines and Choi, 2016).

It is still unclear what host family *Peronospora* evolved on, but it is hypothesized that the presence of *Peronospora variabilis* on quinoa is the result of a host jump from a weedy relative, most likely *Chenopodium album* (lambsquarters), however this hypothesis has not been proven (Thines and Choi, 2016).

It is important to note the complicated taxonomy of fungi and oomycetes. There are several taxonomic terms to distinguish organisms below the level of species. Races and forma specialis (special forms) are common taxonomic terms used to identify fungi, bacteria, and oomycetes to a highly specific level based on pathogenicity. Races of pathogens are defined as organisms that differentially infect different cultivars of one species, and special forms are organisms that differentially infect different species within one genus (Lapage et al., 1975). There are hypotheses that there are special forms of *P. variabilis* that are highly host species specific and only infect certain species within the *Chenopodium* genus. There have been cross-infection studies performed by researchers reporting *P. variabilis* host-specificity within the *Chenopodium* genus however further research is important in order to further support these reports (Risi and Galwey, 1984; Tewari and Boyetchko, 1990; Byford, 1967, Kumar et al., 2006).

The epidemiology and genetics of this fungal species is still widely unknown as it has only recently been recognized as an important species within the past twenty years (Thines and Choi, 2016). Taking a step back to evaluate the entire genus, many aspects of *Peronospora* biology and evolution are largely unknown, and it is still unclear as to how many species of *Peronospora* actually exist. *P. variabilis* and the *Peronospora* genus pose many challenges to researchers trying to fill this wide knowledge gap: there are relatively few scientists investigating these organisms, investigations have been limited to the temperate zones of North America and

Europe, *Peronospora* spp. have extremely complicated taxonomy, and it is unknown if the geographical ranges of pathogens and hosts are congruent (Thines and Choi, 2016).

Evidence of fungicide resistance of P. variabilis

Heavy use of fungicides and other chemicals to control plant diseases places a selective pressure on the pathogen, leading to the evolution of fungicide resistance. This process happens relatively quickly in sexually reproducing pathogens and these organisms thus have a higher risk of developing fungicide resistance compared to asexually reproducing pathogens. An important fungicide class in controlling fungal plant pathogens are quinone outside inhibiting (QoI) fungicides, which inhibit the cytochrome bc complex of mitochondrial respiration (Chen et al., 2007). This class is effective against oomycete, basidiomycete, and ascomycete fungi, which are three major groups of plant pathogens (Chen et al., 2007). Soon after their introduction in 1996, QoI fungicide resistant isolates were found in multiple pathogen populations (Chen et al., 2007). This fungicide class is site-specific, specifically targeting the Qo site of the cytochrome bc1 enzyme complex, and generally carries a high risk of resistance development, as shown by reports of resistant strains of grapevine and cucumber downy mildews, as well as *Alternaria solani* (tomato blight), *Puccinia* spp. (rust fungi), and *Venturia ineaqualis* (apple scab) (Chen et al., 2007; Ishii, 2006).

In a 2002 study investigating cucumber pathogens, it was found that resistant strains of cucumber downy mildew (*Pseudoperonospora cubensis*) were predominant in a greenhouse three years after the use of the fungicide was discontinued (Ishii et al., 2002). Additionally, just two years after QoI fungicides were introduced in France, fungicide resistance was detected within *Peronospora viticola* populations among most French vineyards (Chen et al., 2007). It has been found that in most cases this resistance is due to a single point mutation in the

mitochondrial cytochrome b gene resulting in a change in amino acid from a glycine to alanine at position 143 (G143A) (Chen et al., 2007). Currently, many farmers use fungicide mixtures or rotations to combat fungicide resistance (Hideo, 2006). However, according to a simulation model by Dekker (1982), this approach may only be successful in delaying fungicide resistant rather than stopping the development altogether (Hideo 2006, Dekker 1982).

Evidence of fungicide resistance in other downy mildew pathosystems as well as the little research and knowledge of fungicide resistance in *P. variabilis* populations gives reason to be cautious. The issue of fungicide resistance is important to consider ahead of time as it can evolve quickly and have drastic effects on the effectiveness of fungicides being used. The issue of fungicide resistance raises the point of the importance of an integrated approach to disease management. While this study focuses on the investigation of disease resistance, this will not be sufficient as a standalone strategy to mitigate quinoa downy mildew in New England. Disease resistant plants combined with fungicides, biocontrols, and proper cultural and sanitation strategies will provide the most protection against diseases in future New England production of quinoa.

Host resistance for management of quinoa downy mildew

Employing genetically resistant crops in agriculture can have many advantages including reduction of fungicide dependence, cost savings associated with buying these preventative chemicals, and reduction in labor costs. Downy mildew diseases are extremely prolific around the world and have been reported as economically significant diseases on many crops. Three pathosystems in which the resistance mechanisms to downy mildew has been relatively well studied are *Arabidopsis thaliana – Hyalonospora arabidopsis*, *Ocimum basilicum* (basil) – *Peronospora belbahrii*, and *Spinacea oleracea* (spinach) – *Peronospora effusa*. Several

resistance genes have been reported in *A. thaliana* (*RPP* genes), basil (*Pb*1 genes), spinach (*Pfs*-1 locus), as well as other species affected by the disease (Botella et al., 1998; Ben-Naim et al., 2018; Irish et al., 2008). Thus far researchers have the most knowledge of the *A. thaliana* RPP genes, which encode proteins with nucleotide binding, leucine-rich repeat (NBLRR) motifs (Nemri et al., 2010). Resistance to downy mildew in *Arabidopsis* has been seen to be primarily attributed to single dominant resistance genes as the most common mode of resistance, with additive and epistatic resistance playing minor roles (Nemri et al., 2010). It is still unknown whether or not these known downy mildew resistance genes are highly similar in sequence similarity and if they overlap in function. It is possible that single dominant R genes play a bigger role in certain downy mildew pathosystems and additive resistance contributes more to resistance in others. While there are many unknowns, the common presence of these genes in other downy mildew systems suggest that quinoa and other species within the *Chenopodium* genus have or could acquire similar downy mildew resistance genes that have yet to be found and confirmed. While this aim is beyond the scope of this thesis, my research provides the preliminary background for future genetic and bioinformatic experiments delving into the presence of downy mildew resistance genes within the *Chenopodium* genome.

The Chenopodium genus

Host systematics

The genus *Chenopodium* is in the Caryophyllales order and Amaranthaceae family. There are 100-150 species reported within the genus and these are distributed mainly in Eurasia, North America, and South America (Jellen et al., 2011; Neff, 2017). The species within the genus vary greatly in ploidy levels and subgenome composition (Figure 1-6, Walsh et al., 2015). Quinoa is an allotetraploid with a sporophytic chromosome number of 2n = 4x = 36 (Palomino et al.,

2008). The complexity and large genome size (1C =1146-1503 Mbp) of this species poses many challenges to genetic and genomic studies of the crop (Palomino et al., 2008; Kolano et al., 2008).

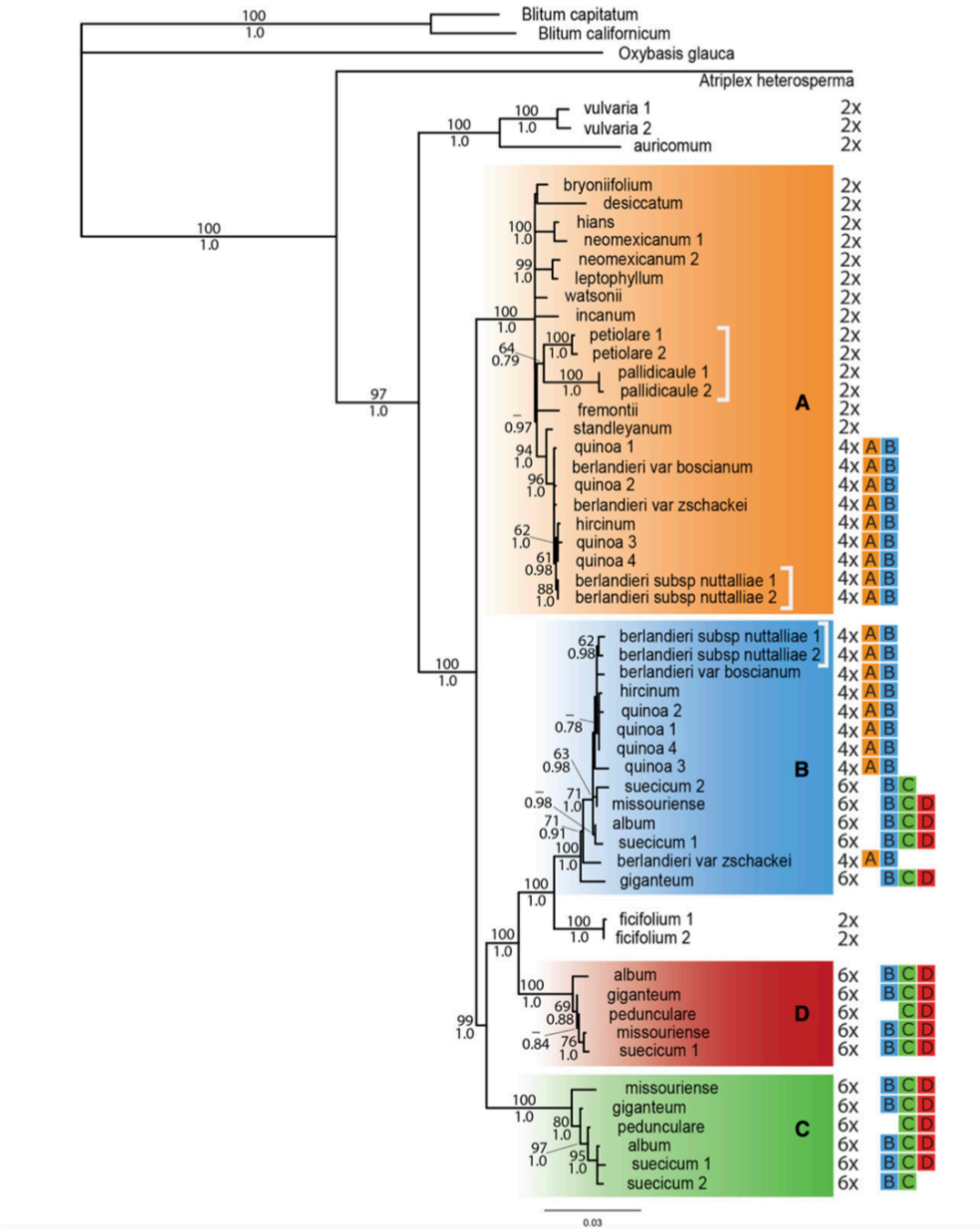

Figure 1-6. A molecular phylogeny of the *Chenopodium* genus based on SOS1 (*Salt Overly Sensitive* 1) sequencing from Walsh et al. 2015. The phylogeny also displays the ploidy of each species, as well as its inferred subgenome composition.

New England native species

There are 11 reported native or naturalized *Chenopodium* species in New England: *C. ficifolium*, *C. berlandieri* var. *macrocalycium*, *C. berlandieri* var. *bushianum*, *C. berlandieri* var. *zschakei*, *C. album*, *C. strictum* var. *glaucophyllum*, *C. standleyanum*, *C. foliosum*, *C. fogii*, *C. pratericola,* and *C. leptophylum* (Neff, 2017). This list was created by cross-referencing 23 *Chenopodium* species from NNE herbarium records (NEHerbaria.org) against 17 chenopod species described in Arthur Haines' Flora Nova Angliae (2011, pp.321-325), creating a new, reduced list of taxa that was then compared to recent phylogenetic studies of the genus (Neff, 2017).

Economic uses

Quinoa is the most economically important species of the genus *Chenopodium*, and its high nutritive properties have resulted in it being used to make flour, soup, used as a rice substitute in many dishes, and as fodder for cattle (Bhargava et al., 2006; Galwey, 1989). It can also be used in alcohol fermentation, and many brewers have created quinoa-based beers. Quinoa is also an effective food substitute for people with celiac disease, as it does not contain gluten (Bhargava et al., 2006). Few instances of medicinal applications have been reported, it has been used to treat inflammation, as an analgesic, and a disinfectant of the urinary tract (Barghava et al., 2006). It has also been reported to be used to treat internal hemorrhaging as well as an insect repellent; these few reports of medicinal properties suggest other possible economically viable avenues for use of quinoa (Mujica, 1994; Bhargava et al., 2006).

Beginning in the 1980's quinoa has been considered for use in NASA's Controlled Ecological Life Support System (CELSS) because of its starch content that is highly suitable for the emulsion of food products (Ahamed et al., 1996). CELSS aims to use plants to remove

carbon dioxide from the atmosphere to generate food, water, and oxygen for astronaut crews for long-term space missions, and more recently, has been experimenting with growing food in outer space (Schlick and Bubenheim, 1996). In 2015, quinoa was approved as a "bonus food" by NASA and has been eaten by astronauts on board the International Space Station (FAO, 2015). Quinoa is an excellent candidate crop and food source for space travel, as it has been seen to respond well in a hydroponic environment and is able to be stored at room temperature for longer periods of time (FAO, 2015).

Nutritional content

Quinoa is well known for its high nutritional content; it is high in vitamins, minerals, and amino acids. Its protein content has been found to be around 15% by weight on average, considerably higher than most other grains (USDA, 2015). Quinoa is one of the few plants that provides all essential amino acids, making its consumption extremely popular in populations with food insecurity with little access to a diverse and complete diet (Navruz-Varli and Sanlier, 2016). The plant's carbohydrate content is mainly starch, and it is very rich in essential fatty acids like linoleic acid (Navruz-Varli and Sanlier, 2016). In regard to vitamin content, little research has been done, but according to a 2009 study (Abugoch James, 2009), the riboflavin content in 100g of quinoa seed is enough to meet 80% of children's needs and 40% of adult's vitamin requirement.

Ash content (3.4%) is higher than in rice (0.5%), wheat (1.8%) and many other grains (Navruz-Varli and Sanlier, 2016). Due to its high ash content, quinoa contains large amount of minerals and its iron and calcium content are higher than in most other grains (Navruz-Varli and Sanlier, 2016). Quinoa also contains phytochemicals such as saponins, these molecules negatively affect taste and digestibility and are typically removed before consumption; however,

they have been associated with antifungal, antiviral, diuretic, hypoglycemic, and anti-inflammatory properties (Navruz-Varli and Sanlier, 2016).

Objectives and aims

The overall goal of this project was to identify potential pathogens infecting quinoa in New Hampshire and investigate genetic resistance to downy mildew among *Chenopodium* species native to New England. Specific objectives were to;

1. optimize molecular protocols to detect *Peronospora variabilis* from quinoa tissue and confirm the presence of the pathogen in New Hampshire,
2. evaluate differential resistance to disease among *Chenopodium* species and accessions in a field setting and evaluate the severity and spread of disease,
3. investigate the molecular relationships among *P. variabilis* isolates from different *Chenopodium* hosts,
4. identify other potential pathogens of *Chenopodium* spp. in New England, including a stem lesion disease first observed in New Hampshire on *Chenopodium* spp.

Outcomes and outputs generated from this research will provide a framework for future *Chenopodium* disease resistance studies to investigate the genetic basis of downy mildew disease resistance, differential susceptibility within the genus, and molecular evolution of the *P. variabilis* organism. Addressing these gaps in knowledge is integral to the overall goal of developing management practices for production of quinoa in New England.

CHAPTER TWO: CONFIRMATION OF *PERONOSPORA VARIABILIS* ON *CHENOPODIUM* SPECIES IN NEW HAMPSHIRE

INTRODUCTION

Downy mildew of quinoa, caused by *Peronospora variabilis*, has been reported in every country where quinoa is cultivated (Danielson et al., 2003). This pathogen not only infects quinoa but other weedy relatives in the *Chenopodium* genus; however, it is specific to *Chenopodium* species (Thines and Choi, 2016). *P. variabilis* is a fungal-like organism that causes areas of chlorosis and pink discoloration on the leaves leading to defoliation and reduction of plant vigor. The disease can reduce seed yield by 33% to 99% in highly susceptible quinoa varieties (Testen et al., 2014). The presence of oospores among Pennsylvania isolates and two different mating types were confirmed (Testen et al., 2014). *P. variabilis* is thought to be heterothallic, requiring two different compatible mating types for sexual reproduction to occur and production of oospores (Danielsen, 2001). When two compatible mating types are not present within a region, the pathogen is limited to asexual reproduction. This can greatly affect the population genetics of the pathogen and thus the amount of genetic variability and recombination of a *P. variabilis* population in a certain area. *P. variabilis* was originally thought to be endemic to South America, but as of 2012 the pathogen has also been reported on *C. quinoa* in the United States in Lancaster County, PA (Testen et al., 2014).

Recently, downy mildew symptoms were observed by a UNH graduate student, Erin

Neff, on multiple *Chenopodium* species in Durham, NH in 2016 and 2017. Symptoms were first observed on quinoa field trials at the University of New Hampshire's (UNH) Woodman Farm in 2016. The disease was diagnosed as downy mildew by the UNH diagnostic lab based on appearance of symptoms and presence of the characteristic gray-purplish sporulation on the underside of the leaves, but the causal agent was not confirmed using microscopic or molecular diagnostic methods. The presence of *P. variabilis* has posed a significant obstacle to the overall goal of redomesticating quinoa in New England as a commercial crop.

Since this pathogen has not yet been reported in New Hampshire or the surrounding New England area, many aspects of its local epidemiology are still unknown. The objective of this study was to confirm the presence of *P. variabilis* on *Chenopodium* species at Woodman Farm. A secondary objective was to investigate the source of infection, specifically to determine if the infection was introduced from planting initially infected seed, or if *P. variabilis* was already established in the area.

METHODS

Field site and site history

This study used plant material growing at the UNH Woodman Research Farm located in Durham, NH. Harvested seed was collected from accessions planted in 2017 at Woodman Farm in raised beds with black plastic mulch (Dodges Agway, Exeter NH). The plot's soil type is classified as Woodbridge series, consisting of sandy, loamy soils (Evan Ford, personal communication). During the previous 2016 growing season, the plot was left fallow and, in the fall of 2016, winter rye was planted as a cover crop. Since 2016, only *Chenopodium* species have been planted in the plot used, excluding the winter rye cover crop grown during the off-season.

Downy mildew-like symptoms were observed on *Chenopodium* species planted for the first time in 2016, but protocols to confirm the pathogen were not performed. The plot used has been managed organically for many years (Evan Ford, personal communication). During the 2017 growing season at Woodman Farm, a disease suspected to be downy mildew was observed on several *Chenopodium* species.

Plant material

Chenopodium spp. seed used in this experiment was obtained from a previous Master's student in the UNH Davis lab, Erin Neff. During the 2017 growing season at Woodman Farm, seed from severely diseased, moderately diseased, and non-diseased plants was harvested. Seed from the seed stock of the different accessions was also obtained in order to investigate whether the seed was already infected before planting. The seed stock collections were stored in envelopes in a cool, dry temperature-controlled laboratory. Additional plant material was obtained from the USDA National Plant Germplasm System (NPGS) or was collected from sites throughout New England (Table 2-1). Material used in this study was collected by Erin Neff.

Table 2-1. *Chenopodium* accessions tested for *P. variabilis* infection in this study. For seed received from the USDA National Plant Germplasm System (NPGS), accession numbers are listed. Each accession has a shorthand collector code by which it is referred.

		Material tested		
Chenopodium species/Collector code	Source	Field seed	Stored seed	Leaf tissue
C. berlandieri var. *macrocalycium* x *C. quinoa* / BYU 803	Brigham Young University		x	
C. berlandieri var. *macrocalycium* x *C. quinoa* / BYU 803F	Harvested BYU 803 Seed - Woodman Farm 2017	x		
C. berlandieri var. *macrocalycium* / BVM Rye	Collected - Rye Beach, NH		x	
C. berlandieri var. *macrocalycium* / BVM Rye F	Harvested BVM Rye Seed - Woodman Farm 2017	x		
C. berlandieri var. *macrocalycium* / BVM USDA	USDA NPGS - PI 666279	x		
C. berlandieri var. *macrocalycium* / BVM Appledore	Collected - Appledore Island, ME	x		
C. quinoa cv. 'Brightest Brilliant Rainbow' / Rainbow	Wild Garden Seed		x	
C. quinoa / 37P	USDA NPGS - Ames 13734	x		
C. quinoa / QQ065	USDA NPGS - PI 614880	x		
C. album / CA286	Collected - Ogunquit, ME		x	
C. album / CAW-1	Collected - Woodman Farm			x
C. album / CAME	USDA NPGS - PI 666272	x		

Microscopic identification of P. variabilis

In October 2017, a *C. album* plant suspected to be infected with downy mildew was observed at Woodman Farm. The plant was showing symptoms of yellow leaf discoloration and had gray sporulating lesions on the underside of leaves. Five to seven symptomatic leaves were collected from Woodman Farm and placed in a plastic bag with a damp paper towel and kept in a 4°C refrigerator until used for microscopy. Additionally, the whole plant was dug up from the farm and transplanted into a potting mix and kept at the UNH Macfarlane Greenhouse. Wet mounts were made by scraping sporulating lesions off of the collected leaf surface with a sterile dissecting needle and placing the collected sample in reverse osmosis (RO) water on a glass slide. A cover slip was placed over the sample and microscopy was performed with an Olympus BX40 microscope (Olympus Corporation of the Americas, Center Valley, PA) using the 40X objective. Microscopy was performed to detect diagnostic morphological features of *Peronospora variabilis* such as sporangiophores, sporangia, and oospores. After *P. variabilis* was confirmed microscopically, a stage micrometer was used to record sporangia and sporangiophore dimensions at the 40X objective.

Detection of P. variabilis in seed

Seeds were soaked in potassium hydroxide (KOH) to increase DNA extraction yield through softening of the seed coat and releasing oospores and sporangia within the endosperm (Danielsen et al., 2004). One hundred seeds of each sample (Table 2-1) were soaked in 20 ml of 1N KOH for 16 hours to break down the endosperm and loosen and soften the seed coat (Danielsen et al., 2004). Wet mounts were prepared using the KOH soaked seed. Microscopy was performed to detect oospores and other spore structures released from the seed coat. Once KOH soaks were complete, seeds were rinsed three times over with sterile milliQ water in order

to prevent chemical interaction throughout the DNA extraction process. When DNA from KOH soaked samples were extracted, soaked seeds were first frozen with liquid nitrogen and ground up using a mortar and pestle before continuing with the DNA extraction protocol described below.

DNA extractions

To confirm the presence of the pathogen using molecular methods, DNA extractions were performed on KOH-soaked seeds ground with a mortar and pestle or washed seeds. For non-KOH soaked seeds, before DNA extractions were performed, and a seed wash protocol was used to wash sporangia, sporangiophores, and oospores off *Chenopodium* seed. In this protocol, at least 1 g seed was washed with 10-30 ml milliQ water for 30 minutes at 900 RPM (VWR 7x7" CER Hotplate/Stirrer 120V Pro). Smaller volumes of water were preferred for an increased concentration of the resulting sporangial solution. The suspension was then filtered through sterile cheesecloth, and the resulting solution was centrifuged for 5 minutes at 14,000 x g to obtain a pellet. DNA extraction yields using a cetyltrimethylammonium bromide (CTAB)-based technique (Torres et al., 1993) and a Qiagen DNeasy Plant Mini Kit were compared to determine which protocol would yield more DNA.

The following DNA extraction protocol (Torres et al., 1993) was then used to isolate DNA: 1 mL grinding buffer per sample was prepared by adding 4 ul B-mercaptoethanol to 1 mL 2% CTAB buffer (Appendix I). In a microfuge tube containing the pellet, liquid nitrogen was added and allowed to evaporate, and then the pellet was ground with a pellet pestle. Before the pellet thawed, 1 mL of grinding buffer was added, and the tissue was ground into a slurry. 800 ul of this slurry was then added to a clean 1.5 mL microfuge tube. Once all the samples had been ground, the tubes were incubated in a 60°C water bath for 30 minutes. After incubation, the tubes

were allowed to cool for 10 minutes on the bench. Chloroform:octanol (24:1) was added to nearly fill each tube, and each tube was vortexed until the solution was uniform. The tubes were then centrifuged at 14,000 x g for 5 minutes to separate the phases. Following centrifugation, the upper aqueous phase was transferred to a new 1.5 mL microfuge tube. If the aqueous solution was cloudy, the chloroform extraction steps were repeated. Cold 95% ethanol was then added to each tube with the aqueous phase, and the tubes were stored in a -20°C freezer overnight to increase the amount of precipitated DNA.

On the second day, the tubes were centrifuged at 14,000 x g for 5 minutes, and the supernatant was removed. 1 mL of 70% ethanol was added to each tube and the tubes were kept in the freezer for at least 1-2 hours or overnight. On the third day, tubes were centrifuged at 14,000 x g for 5 minutes and the supernatant was removed. DNA was then dried by placing the open tubes in a speed vacuum centrifuge at 45°C for 1 minute. After drying, 50 ul TE buffer was added to each tube, and the tubes were refrigerated overnight. On the fourth day, a 1 ul RNase/1 mL sterile water solution was prepared, and 50 ul of the solution was added to each tube. The tubes were gently mixed and then incubated at 37°C for 1 hour. Lastly, DNA was quantified with an Invitrogen Qubit fluorometer broad range (BR) kit (Appendix II) and Nanodrop Spectrophotometer.

PCR and gel electrophoresis

After DNA was successfully isolated, touchdown polymerase chain reaction (PCR) was performed according to the following thermocycler profile: initial denaturation step at 94°C for 2 min; followed by 32 cycles of touchdown PCR with denaturation at 95°C for 30 seconds, annealing from 66 to 56°C for 45 s (1 step per degree change and 23 cycles at 56°C), and elongation at 72°C for 90 s, with a final elongation step of 72°C for 5 min (Testen et al., 2014).

For this region, 25-ul PCR reactions were performed with GoTaq green master mix (New England Biolabs), 0.2 uM of each primer, and 5 ul of template DNA. DNA quantification was completed with both a Qubit Fluorometer and Nanodrop Spectrophotometer however, DNA yields were shown as "too low (less than 0.01 ng) or were grossly misestimated due to the presence of residual buffer, respectively. Because of these results, the true amount of DNA used in the PCR reaction is unknown. PCR was performed with forward and reverse PV6 primers (Figure 2-1) developed by Testen et al (2014) using internal transcribed spacer (ITS) sequences from RS and LV *P. variabilis* lines in GenBank following amplification using primer pair DC6/ITS4 (Cooke et al., 2000; Testen et al., 2014) (Table 2-2).

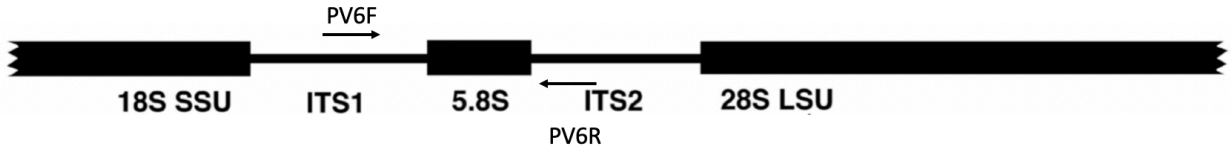

Figure 2-1. Diagram of PV6 primer positions in relation to rDNA structure. Adapted from Raja et al. (2017)

Table 2-2. List of primers used in this study.

Primer name	Region	Sequence (5' to 3')	Source
PV6 Forward	ITS	GTTGCTGGTTGTGAAGGCTG	Testen et al., 2014
PV6 Reverse	ITS	ATGCTACGCAACCGAAGTCA	Testen et al., 2014

Successful amplification was confirmed via visualization of bands with a 1% agarose gel electrophoresis stained with ethidium bromide (Appendix III). Two negative controls were used, one with uninfected *C. album* DNA as the template, and one reaction with no DNA template added. Electrophoresis was used to visualize whether samples produced a ~300 bp band that is indicative of the presence of *Peronospora variabilis*.

DNA sequencing and BLAST analyses

Confirmed amplicons were sent to GeneWiz, LLC (South Plainfield, NJ) for Sanger sequencing services. Resulting sequences were run through the NCBI Basic Local Alignment Search Tool (BLAST) using BLASTn with all default parameters against all *Peronospora* sequences in the database to determine whether the sequences were likely to be those of *Peronospora variabilis* (Altschul et al., 1990).

Downy mildew inoculations

Whole plant inoculations

Whole plant inoculations were performed using pipette and spray protocols. Downy mildew inoculum was obtained on August 15, 2018 from sporulating leaves of *C. ficifolium*, *C. quinoa* var. *negra*, *C. quinoa* R132 (PI 478418), 37P, BVM Rye, BYU 803F, and QQ065 plants at Woodman Farm. Inoculum was produced by using a pipette to scrape sporangia off the leaf material into 3 ml sterile water and adding 1 ul Tween 20 (VWR) and vortexed. This solution was then diluted to 10 ml with sterile water and vortexed again. A water control was created using 10 ml sterile water and 1 ul Tween 20. Two leaves on each of CAW-1, BVM Apple, 37P, QQ065, *C. quinoa* cv. Faro, and BVM Rye seedlings were pipette inoculated with 20 ul of inoculum solution. These inoculated seedlings were kept in a greenhouse humid chamber created with PVC pipe, plastic tarp, and mist nozzles with the tarp up (~75% humidity) for 24 hours and then the tarp down (~99% humidity) for 7 days (Figure 2-1).

Figure 2-2. Greenhouse humid chambers used to control relative humidity.

On October 3, 2018 downy mildew was seen on BVM Apple plants at Woodman Farm and infected leaf material was collected for inoculations. An inoculum solution was created as described above but diluted to 50 ml with sterile water. BVM Rye and QQ065 plants growing in a growth room at 22°C and 74% humidity were sprayed with inoculum using a sprayer (Preval, Coal City, IL) and then sprayed with tap water. Vented high domes (25.4 x 50.8 x 15.24 cm, The HC Company, Griffin Greenhouse Supplies) were placed on top of 50-star deep plug trays (5 x 10, T.O. Plastics, Griffin Greenhouse Supplies) to increase relative humidity and overhead lights were turned off for 24 hours.

Detached leaf inoculations

With the same inoculum obtained on October 3, 2018, detached leaf assays were performed. Six BVM Apple leaves obtained from greenhouse plants and two QQ065 leaves obtained from growth room plants were surface sterilized using 70% ethanol for 10 s and rinsed with milliQ water for 30 s. 20 ul of the sporangial solution was pipetted onto 1% water agar on 100 mm x 15 mm sterile polystyrene plates and sterilized leaves were placed upside down on the solution. Plates were sealed with parafilm and placed in paper bags for 24 hours to block light (Testen, 2012). After 24 hours, plates were taken out of paper bags and subjected to a 16-hour light/8-hour dark photoperiod.

RESULTS

Microscopic identification of P. variabilis on Woodman Farm samples

Microscopic observations of the infected sample supported the initial hypothesis that the collected sample was infected with downy mildew. Sporangia and sporangiophores were observed under the microscope (Figure 2-2). Sporangia were tan in color and averaged 27.5 um in length (n=8, std. dev.=5.7 um) and 17.8 um in width (n=8, std. dev.=2.4 um). Observed sporangiophores were tree-shaped and dichotomously branched at acute angles with attached semi-ovoid sporangia. Some sporangia were still attached to the sporangiophore and some had detached. Sporangiophores averaged 186.5 um in length (n=4, std. dev.=62.5 um) and 7.7 um in width (n=4, std. dev.=2.2 um), with stalk being an average of 111.1 um in length (n=4, std. dev.=53.9 um). Oospores were not seen in any samples.

Figure 2-3. *P. variabilis* sporangium and sporangiophore at the 40X objective isolated from *C. album* collected from the UNH Woodman Farm in August 2017. Photo: H. Nolen.

Detection of P. variabilis on quinoa seed

KOH soaks were performed to visualize oospores as well as to test if softening the seed coat would increase *P. variabilis* DNA extraction yield. KOH soaks were not seen to increase

DNA concentration yields compared to other extraction methods (all KOH soak extractions registered as “too low” for the Qubit to quantify). Additionally, oospores were not found microscopically with or without KOH seed soaks. Sporangia and sporangiophores were seen in both field collected and seed stock samples with confirmed *P. variabilis* DNA.

The CTAB-based technique produced higher yields of DNA than the Quiagen kit and was used for all future *P. variabilis* DNA extractions from seed or leaf tissue. The majority of DNA extraction yields were less than 10 ng, except for 37P (203 ng), CAME (247 ng), and BVM Rye F (280 ng). Samples less than 10 ng were still included in the PCR assay and were seen to still produce bands. PCR and 1% gel electrophoresis showed the diagnostic ~300 bp band on the following samples: BYU 803F, BVM Rye, BVM Rye F, BVM USDA, Rainbow quinoa, 37P quinoa, QQ065 quinoa, and CAME (Figure 2-4). The *C. album* negative control did not produce any clear bands, but there were light streaks within that lane, and the no-template control did not produce any bands. All field harvested seed had confirmed *P. variabilis* DNA while stored seed collections BYU 803 and CA286 did not have *P. variabilis* DNA (Table 2-3).

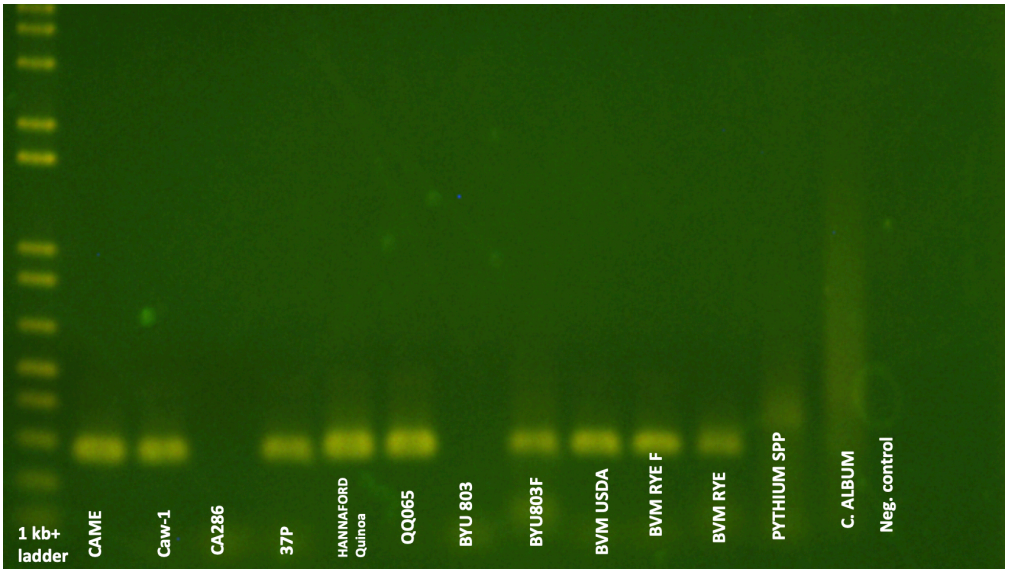

Figure 2-4. Electrophoresis gel showing results of PCR detection of *P. variabilis* in seed washes. Product size was approximately 300 bp.

All sequenced PCR products were confirmed to be *P. variabilis* with average percent identity of 99.2% (n=8, std. dev.=0.582) and an average E score of 1.83 x 10^{-119} (n=8, std. dev.=3.67 x 10^{-119}) (Figure 2-5).

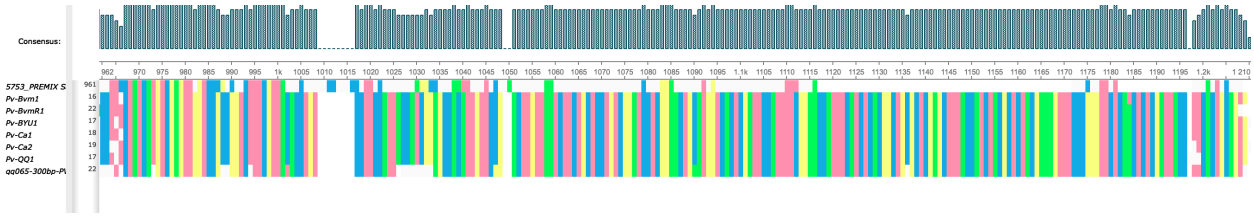

Figure 2-5. Multiple sequence alignment of all PV6 amplicons sequenced in this study.

Table 2-3. *Chenopodium* seed samples that tested for *P. variabilis* DNA. Samples in the left column were harvested from the field and samples in the right column were initial seed stored.

Harvested seed		**Seed stock**	
Collector code	***P. variabilis***	**Collector code**	***P. variabilis***
BYU 803 F	Y	BYU 803	N
BVM Rye F	Y	BVM Rye	Y
BVM USDA	Y	CA286	N
Rainbow	Y		
37P	Y		
QQ065	Y		
CAME	Y		
CAW-1 (leaf)	Y		

Downy mildew inoculations

Neither whole plant nor detached leaf inoculations produced downy mildew disease. For detached leaf inoculations, other secondary organisms were growing on the media. Whole plant inoculations never produced any symptoms or signs of any disease; the average temperature of the greenhouse from August 15 – August 30 was 26°C and humidity was on average 68% (Figure 2-6). The temperatures and humidity level were likely higher under the plastic tarp humid chamber, but precise readings were not recorded. It was observed in August 2018 that the humid chamber temperature exceeded 100°F (37°C) on multiple days during the infection period.

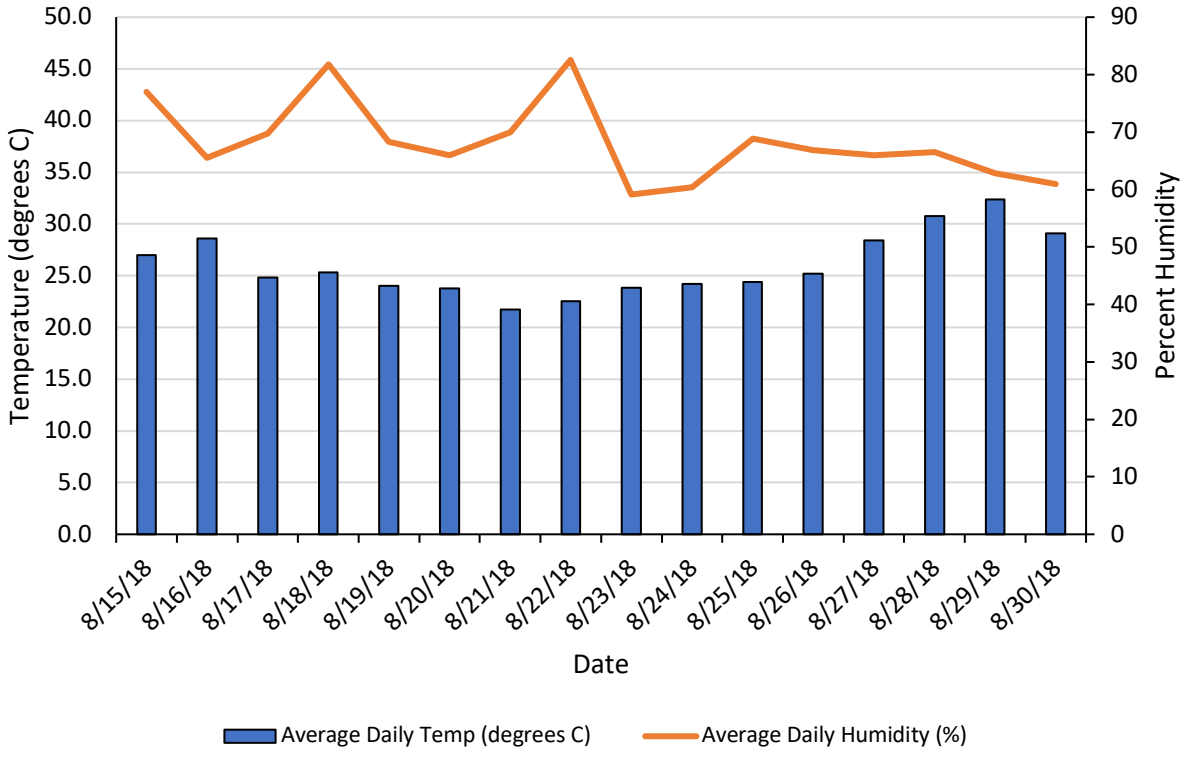

Figure 2-6. Macfarlane greenhouse climate data for August 15, 2018 - August 30, 2018.

DISCUSSION

Microscopic observations of vegetative structures (sporangiophores, sporangia) support the hypothesis that the downy mildew-like symptoms on *C. album* at Woodman Farm were caused by *Peronospora variabilis*. Coupled with the specific host preference of downy mildew, these structures were distinctly characteristic of *P. variabilis*. Observed sporangiophores were tree-shaped and dichotomously branched at acute angles with attached semi-ovoid sporangia that detached from the sporangiophore at maturity, conforming with the diagnostic characteristics of *P. variabilis* (Testen et al., 2012; Danielsen and Ames, 2004; Thines and Choi, 2016). Among all of the seed samples observed microscopically, oospores were not seen with or without KOH seed soaks. Only one isolate from a *C. album* host showed a possible oospore, but due to a dilute sporangial solution, it could not be confirmed if the structure was indeed an oospore. Because oospores were not detected in field samples, it is hypothesized that only one mating type is present in the region, limiting the pathogen to asexual reproduction. Further work is needed to identify what mating type of *P. variabilis* is present in New Hampshire.

P. variabilis was confirmed on all harvested and stored seed samples with the exception of BYU 803 and CA286 (Table 2-3). The methods employed in this study were based off those of Testen et al. (2014), and touchdown PCR and PV6 primers were used in order to get highly specific binding for only *P. variabilis* ITS sequences. Specificity was tested by using these methods with uninfected *Chenopodium* DNA and a *Pythium* spp. sample to ensure PCR was not resulting in the amplification of other fungal DNA. As for the source of infection at Woodman Farm in 2017, it is still unclear the true source of the downy mildew introduction. *Chenopodium* spp. were first planted and seen to be infected with downy mildew in 2016. Without initial seed

planted during the 2016 growing season, it is not possible to pinpoint a source of the infection. From tested seed samples in this study, it was seen that some seed (BVM Rye), was already infected with *P. variabilis* before it was planted in the field. It is possible that the initial infection in 2016 was introduced from acquired infected seed, or it may have already been prevalent in the environment, infecting native weedy species. The latter hypothesis seems more probable as the initially infected seed was seed collected from Rye Beach, NH, suggesting that *Chenopodium* plants in that area of New Hampshire have already been infected with downy mildew.

Another objective of this study was to obtain downy mildew inoculum to maintain in the lab and use for controlled inoculations to satisfy Koch's postulates proving that *P. variabilis* was the cause of disease observed in 2017 and publish a first report of the pathogen in New Hampshire. Unfortunately, I was unable to attain this goal. It is hypothesized that lab inoculations were not successful (1) on detached leaves because inoculum was obtained from the field late in the season and contained secondary organisms that readily grew on agar and competed with *P. variabilis* and (2) did not work on whole plant inoculations because leaves were not kept wet enough for hyphae to grow and penetrate the leaf. In future studies, downy mildew inoculum will be collected as soon as it appears in the field in order to avoid the prevalence of secondary microbes in the inoculum solution, detached leaves will undergo a longer ethanol and water rinse before inoculations to prevent secondary microbes, and a dew chamber will be used when performing whole plant inoculations to keep leaves constantly wet to optimize the environment for infection.

Overall, *P. variabilis* was confirmed microscopically from field samples by looking for diagnostic morphological structures and a PCR protocol was optimized for confirming *P. variabilis* molecularly from seed. These protocols will greatly contribute to future work with *P.*

variabilis in the New England area by providing efficient ways to identify the pathogen. There are, however, many questions that arise from these results. Is there only one *P. variabilis* mating type present in New Hampshire? How diverse is the *P. variabilis* population in northern New England? How long has this pathogen been established in the area and how did it get here? These unanswered questions point to a serious lack of knowledge of the pathogen in New England and serve as reasoning to perform future population genetics studies of *P. variabilis* isolates from different geographical locations within New England. To gain more knowledge of epidemiology and the effect of the pathogen on potential quinoa farming, field experiments and collections should also be incorporated into this research to investigate the prevalence and spread of disease caused by *P. variabilis* in the area.

CHAPTER THREE: EVALUATION OF DOWNY MILDEW DISEASE SEVERITY ON *CHENOPODIUM* SPECIES AND ASSESSMENT OF MOLECULAR RELATIONSHIPS AMONG *PERONOSPORA VARIABILIS* ISOLATES IN NEW HAMPSHIRE

INTRODUCTION

Quinoa, *Chenopodium quinoa* Willd., is an Andean crop prized for its high nutritional content and environmental adaptability (Testen et al., 2014). The crop has been gaining attention in the United States and Europe due to its high-quality protein content. Specifically, sulfur rich amino acids, like cysteine and methionine, are present in higher quantities in quinoa than typical cereals such as corn and rice (Bhargava et al., 2006). Quinoa's nutritive properties and environmental hardiness make it a key potential crop for agriculture in North America. However, certain genetic traits such as its susceptibility to downy mildew will likely be important to consider when attempting to introduce the crop to New England. Warm temperatures and high humidity during the New England growing season provide optimum conditions for downy mildew, negatively impacting quinoa growth and yield. Furthermore, because quinoa is a new crop for New England, little is known about what diseases will be most important for growers to know and manage.

Downy mildew, caused by *Peronospora variabilis* is one of the most economically important diseases of quinoa reducing yield up to 33% in tolerant varieties and 99% in susceptible varieties (Testen et al., 2014). *P. variabilis* causes symptoms of chlorosis and pink discoloration on the foliage. Diagnostic signs of the pathogen are gray/black fuzzy sporulation on the underside of the leaf. *P. variabilis* grows within the intercellular space of the leaf tissue and

blocks photosynthetic activity, resulting in the development of necrotic spots and eventual defoliation (Danielsen and Ames, 2004).

Quinoa downy mildew was initially reported as endemic in South America. The disease has now been reported in all areas that quinoa is cultivated, and more recently, the disease has been reported in Canada (Tewari and Boyetchko, 1990) and in the United States in 2012 (Testen et al., 2014) on quinoa plants. As of yet *P. variabilis* causing downy mildew has not been officially reported on lambsquarters or any other weedy relative of quinoa in North America. More specifically, there is no knowledge of the epidemiology and distribution of *P. variabilis* in New England. During the summer and fall of 2017, the downy mildew pathogen was confirmed via microscopic and molecular methods on both *C. quinoa* and *C. album* at the University of New Hampshire (UNH) Woodman Farm in Durham, NH (Chapter Two). This finding suggests that downy mildew will be a major disease challenge for quinoa production in the Northeast.

P. variabilis in the Peronosporaceae family, class Oomycota, phylum Stramenopiles, and kingdom Protista (Thines and Choi, 2016; Dick, 1995). *P. variabilis* is a host-specific pathogen that only infects species in the genus *Chenopodium* including weedy species closely related to quinoa such as *Chenopodium album*, commonly known as lambsquarters (Thines at Choi, 2016). However, there is limited knowledge of this pathogen with respect to its interaction with the host and cross host-species specificity. Some downy mildew researchers hypothesize that *P. variabilis* host specificity is so stringent that there may be different special forms that infect specific *Chenopodium* species (Thines et al., 2009). Several studies have provided evidence of a high-degree of specialization within *Peronospora* (Thines and Choi, 2016; Belbahri et al., 2005; Choi et al., 2008, 2010). Cross-pathogenicity tests have been performed by quinoa downy mildew researchers to investigate the host specificity of the pathogen. Byford (1967) was one of

the first studies to report that downy mildews formerly classified as *P. farinosa* are limited by host range and should be differentiated as such. Additionally, in later studies by Kumar et al. (2006) and Aragaon and Gutierrez (1992) reported that there is no cross pathogenicity of naturally existing isolates of *P. variabilis* across weedy species of *Chenopodium*. These reports suggest differential susceptibility to *P. variabilis* within the genus and the possibility of host species-specific isolates. The epidemiology and genetics of this fungal species is still widely unknown as it has only recently emerged within the past twenty years (Thines and Choi, 2016). The presence of *P. variabilis* special forms has yet to be confirmed or denied. In addition to cross-inoculation studies, phylogenetic analyses can provide great insight to this question to reveal differences among *P. variabilis* isolates at the molecular level. The finding of downy mildew on *C. album* and *C. quinoa* in New Hampshire, provides the opportunity to compare NH isolates of the pathogen infecting *C. quinoa* and its weedy relatives and to study the mechanisms of resistance in different species of *Chenopodium*.

Downy mildew has been reported to drastically reduce yield of quinoa (Testen et al., 2014) and its presence has been documented worldwide, making it the most damaging and widespread disease of quinoa (Danielsen et al., 2004). Like other downy mildew causal organisms, *P. variabilis* is very difficult to manage. In order to control downy mildew, growers traditionally use fungicides such as metalaxyl (Brouwer et al., 2003). Metalaxyl is expensive, provides a selection pressure for pathogens to quickly evolve resistance, and requires multiple applications, greatly increasing labor costs (Aegerter et al., 2002; Brouwer et al., 2003). Its spores can travel long distances via wind and rain. The pathogen can persist within the seed coat (Danielsen et al., 2004), and its sexual spores (oospores) overwinter in soil and plant debris. As a result, the pathogen can remain in the environment in the absence of its host for long periods of

time. Employing genetically resistant crops can have many advantages including reduction of fungicide use, cost savings associated with buying these preventative chemicals, reduction in labor costs, and reduction of selective pressure imposed by fungicides.

There are many examples of confirmed and well-studied downy mildew resistance genes in other pathosystems such as *Arabidopsis thaliana* (*RPP* genes), basil, (*Pb1* genes), and spinach (*Pfs-1* locus) (Botella et al., 1998; Ben-Naim et al., 2018; Irish et al., 2008). The presence of these genes in other downy mildew systems suggests that quinoa and other species within the *Chenopodium* genus may have similar downy mildew resistance genes that have yet to be identified and confirmed within the genome. A common strategy used to identify potential resistance genes or loci is to look for disease tolerance in wild relatives of the crop of interest. Kumar et al. (2006) report several *Chenopodium* accessions immune/resistant to *P. variabilis* that could potentially serve as a source of resistance genes to downy mildew. Kitz (2008) also reports results suggesting the presence of horizontal resistance among quinoa breeding lines with genotypes NL6 and Sayana showing moderate resistance, and 0654 showing the most resistance. This study serves as the first step in the process of identifying resistance genes in *Chenopodium* spp. by evaluating differential resistance to disease among *Chenopodium* species native to New England.

The overall goal of this study was to characterize diseases of *Chenopodium* species in New Hampshire. Specific objectives were to (1) evaluate differential resistance to disease among 10 *Chenopodium* accessions representing four different species, (2) investigate the phylogenetic relationships of *P. variabilis* isolates infecting different *Chenopodium* accessions, and (3) determine what quinoa pathogens are present in New England and likely to be an issue for growers. It was hypothesized that different *Chenopodium* accessions will display differential

levels of susceptibility, suggesting that multiple genes are responsible for disease resistance. Additionally, when considering previous research performed with this pathosystem, it is also possible that there are immune accessions that harbor major resistance. The results of this study will provide information about what *Chenopodium* species are most likely to possess effective disease resistance genes. These species will be included in future studies to investigate the genetic basis of disease resistance in *Chenopodium* and eventually be incorporated into the quinoa breeding program at the University of New Hampshire to develop a quinoa variety that is tolerant to disease and able to be grown effectively in New England.

METHODS

Seed and plant material

Chenopodium seed was obtained from the USDA National Plant Germplasm System (NPGS), previously planted material, and from naturally occurring plants at sites around New England (Table 3-1). In this experiment, three accessions of a *C. berlandieri* var. *macrocalycium* (BVM), two accession of a BVM x *C. quinoa* hybrid, one *C. album* accession, and four *C. quinoa* accessions were tested. BVM accessions were collected from Rye Beach, NH (BVM Rye), Appledore Island, ME (BVM Appledore), and the USDA (BVM USDA PI 666279). The *C. quinoa* accessions var. Faro (Sisikyou Seeds), Rainbow quinoa (Wild Garden Seeds), quinoa 37P (USDA Ames 13734), quinoa QQ065 (USDA PI 614880) were included. The *C. album* was collected from Woodman Farm (CAW-1).

Field study experimental design

This experiment consisted of a 2-factor randomized split plot design with 10 *Chenopodium* accessions (Table 3-1) and two mulching systems (black plastic or none) for a

total of 20 treatments with three replicate plots per treatment. A total of 240 individual plants were planted in six rows, such that three adjacent rows were raised beds with black plastic mulch, and the other three rows plants were directly seeded or transplanted in the ground. Each row was composed of 10 subplots representing each accession composed of four plants. The accession subplots were randomly distributed within each row. Plants within a subplot were 2.5 feet (76.2 cm) apart, and subplots were 4.5 feet (137.16 cm) apart, with rows being approximately 118 feet (35.96 m) long.

Table 3-1. *Chenopodium* accessions evaluated in the field study in 2018. For seed received from the USDA National Plant Germplasm System (NPGS), accession numbers are listed. Each accession has a shorthand collector code by which it will be referred.

Chenopodium species	Collector code	Source	USDA Accession #
C. berlandieri var. *macrocalycium* x *C. quinoa*	BYU 803	Brigham Young University	-
C. berlandieri var. *macrocalycium* x *C. quinoa*	BYU 803F	Harvested BYU 803 Seed - Woodman Farm 2017	-
C. berlandieri var. *macrocalycium*	BVM Rye	Collected - Rye Beach, NH	-
C. berlandieri var. *macrocalycium*	BVM USDA	USDA NPGS	PI 666279
C. berlandieri var. *macrocalycium*	BVM Appledore	Collected - Appledore Island, ME	-
C. quinoa cv. 'Faro'	Faro	Sisikyou Seeds	-
C. quinoa cv. 'Brightest Brilliant Rainbow'	Rainbow	Wild Garden Seed	-
C. quinoa	37P	USDA NPGS	Ames 13734
C. quinoa	QQ065	USDA NPGS	PI 614880
C. album	CAW-1	Collected - Durham, NH	-

Field preparation and planting

This study was conducted at the UNH Woodman Research Farm in Durham, NH from June 21, 2018 to September 16, 2018. In the previous growing season (2017), the plot was left fallow and, in the fall of 2017, winter rye was planted as a cover crop. Prior to planting this experiment, the winter rye was incorporated into the soil with a moldboard plow and disk harrows were used to break down large soil clods (Evan Ford, personal communication). Raised beds were prepared with a Reddick raised bed former and a 65-horse power John Deere 5325 tractor using 1.25 mil black plastic (Dodges Agway, Exeter NH). The plot's soil type is classified as Woodbridge series, consisting of a sandy-loam soil type. Irrigation was applied using Riviulis t-tape (Rain-flo Irrigation, 508-12-450) drip line running under the plastic for raised beds and on the soil surface for non-raised beds. The drip line flow rate was kept at a constant .45 GPM/100 ft (Evan Ford, personal communication). Two of each accession in every row were direct seeded on June 21, 2018 and the remaining plots were planted on June 28, 2018 with transplants grown at the MacFarlane Greenhouse.

Plot history

Since 2016, the experimental plot has been planted with *Chenopodium* species as a part of the Davis lab quinoa research program, excluding the winter rye cover crop grown during the off-season. The larger plot has been divided into two subplots, which rotate use every growing season. During the previous 2017 growing season, the experimental plot was held fallow, with the adjacent plot consisting of various *Chenopodium* species.

Disease assessment

Downy mildew disease onset, disease incidence, and disease severity measurements were collected over the field season. Disease severity assessments were performed on July 27, August

3, August 17, September 1, and September 15. Each plant was scored based on the following scale:

0 = no downy mildew symptoms
1 = yellow discoloration of leaves
2 = light sporulation only visible with hand lens
3 = light sporulation visible with naked eye
4 = moderate sporulation
5 = heavy sporulation and/or defoliation

Disease evaluations were performed by the same person to ensure consistency. Evaluations were based on assessing individual plants on three levels: (1) disease severity on the 0-5 scale, (2) percent of plant affected, and (3) presence or absence of pink discoloration. Downy mildew symptoms differed among *Chenopodium* accessions: in some species a pink discoloration (Fig 3-1) was observed within the lesions in addition to sporulation and chlorosis. The presence or absence of the pink discoloration was recorded for each date, and incidence data from July 27, 2018 were analyzed to determine if this symptom was correlated with certain species or accessions. Pink discoloration incidence was calculated using the equation below:

$$Pink\ discoloration\ incidence = \frac{number\ of\ plants\ in\ subplot\ with\ discoloration}{total\ number\ of\ plants\ in\ subplot} * 100$$

Figure 3-1. Pink discoloration symptoms observed in 2017-2018. Symptoms are shown on BYU 803 F accession. Photo: H. Nolen.

Area under the disease progress curve

To quantify the disease intensity over time, the area under the disease progress curves (AUDPC) was calculated for downy mildew severity and overall disease severity over time for each accession. The following equation was used to calculate AUDPC (Shaner and Finney, 1977):

$$AUDPC = \sum_{i=1}^{n}((y_i + y_{i+1})/2) * (t_{i+1} - t_i)$$

Where n is the number of assessments, t_i is the sample time point, and Y_i is the measured disease severity. The most common way to calculate AUDPC is the trapezoidal method in which the area under the disease progress curve is calculated by breaking the area into a series of rectangles (Figure 3-2), calculating the area of each rectangle (l x h), and adding the areas of the rectangles. In this study, AUDPC values were calculated per accession. There was a disease severity value for each time point in which disease severity was evaluated, starting at t = 0, y = 0.

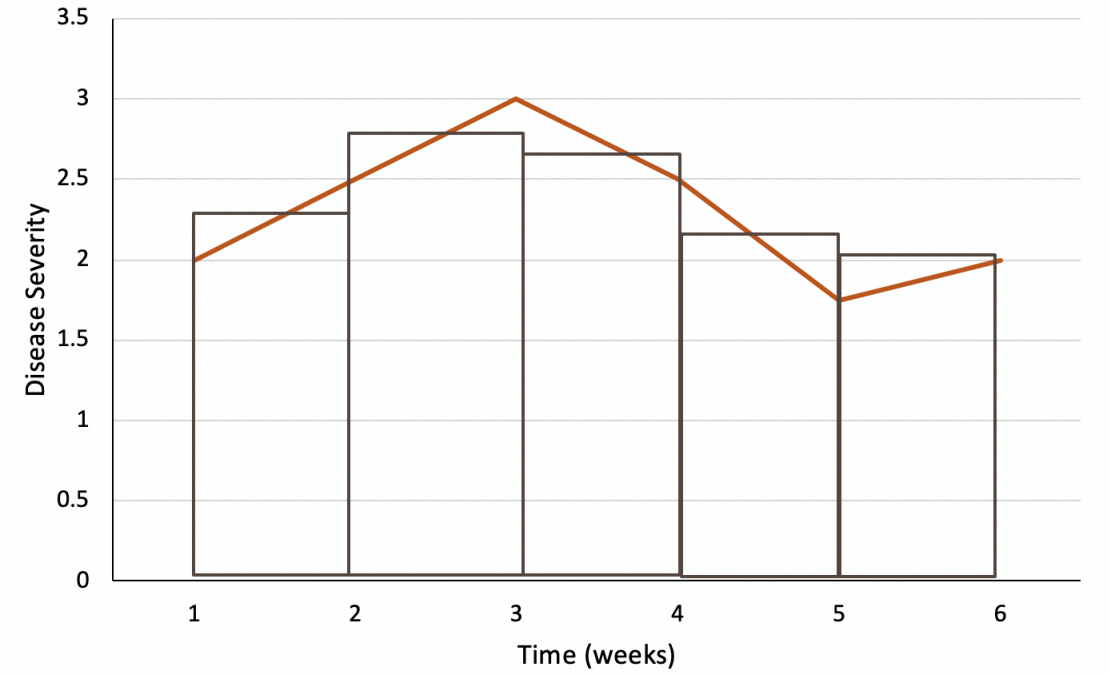

Figure 3-2. Graphical depiction of the AUDPC calculation in which one replicate of each accession is evaluated at a time, and the area under the disease severity over time curve is calculated using the trapezoidal method.

Data analysis

AUDPC values and disease severity data were analyzed using a two-way analysis of variance (ANOVA). To compare disease severity among treatments, a repeated measures ANOVA with a residual covariance structure was performed using JMP 14 software and significance was assessed at $\alpha = 0.05$. The model statement for disease severity consisted of accession, mulch, row and accession x mulch, with the individual plant as the experimental unit. Row (block) was considered as a random effect in all analyses. The AUDPC ANOVA was also performed using JMP 14 software at $\alpha = 0.05$. For both downy mildew severity and overall disease severity, post-hoc tests were done using mean separation Tukey Kramer tests. In addition to Tukey Kramer tests, Kruskal-Wallis tests were also performed on downy mildew severity data for each date. For pink discoloration incidence, presence/absence data were used to calculate an

incidence value, the calculations used can be seen below. Incidence values from July 27, 2018 were then analyzed via ANOVA at $\alpha = 0.05$.

Phylogenetic analyses of downy mildew isolates

For each accession, 4-5 sporulating leaves were collected from two plants located in different subplots. Collected leaf tissue was placed in plastic Ziploc bags with a damp paper towel and held on ice until brought back to the lab. Samples were kept separate and were not combined into composite samples. Infected leaf material was kept in a refrigerator until DNA extractions were performed. To prepare seeds for DNA extraction, at least 1 g of seeds were washed in 10 ml of sterile milliQ water and stirred for 30 min at speed setting 900 RPM (VWR 7x7" CER Hotplate/Stirrer 120V Pro), the resulting solution was filtered through two layers of sterile cheesecloth and centrifuged for 5 min at 14,000 RPM . To prepare infected leaf tissue, sporulating areas were cut from the leaf with a sterile blade and these sporulating lesions were placed in a 1.5 ml microcentrifuge tube and washed with 1 ml sterile milliQ water by vortexing for 30 to 90 seconds. After vortexing, infected lesions were scraped with a sterile pipet tip to dislodge sporangia into the solution. The leaf wash solution was then centrifuged at 14,000 RPM for 5 minutes to obtain a pellet. DNA was then extracted from the seed and leaf wash was solutions by cetyltrimethylammonium bromide (CTAB) DNA extraction, using liquid nitrogen to break open cells as described by Torres and colleagues (1993).

After DNA was successfully isolated and quantified, the presence of *Peronospora variabilis* was confirmed using the molecular identification protocol described in Chapter Two. For phylogenetic analyses, the cytochrome oxidase subunit 2 (COX2) region of the isolated *P. variabilis* DNA was amplified using polymerase chain reaction (PCR) according to the following thermocycler profile: initial denaturation step at 96 °C for 4 min; followed by 30 cycles with

denaturation at 96 °C for 30 s, annealing from 47.2 °C for 30 s, and elongation at 65 °C for 60 s, with a final elongation step of 65 °C for 4 min (Cooke et al., 2000). PCR was performed with forward and reverse Peronosporomycete primers developed by Hudspeth et al. (2000) based on aligned complete COX2 sequences of *Phytophthora megasperma* (Sachay et al., 1993). For this region, 25-ul PCR reactions were performed with Long Amp Taq Polymerase (New England Biolabs), 1X Long Amp buffer (New England Biolabs), 0.2 uM of each primer, and 5 ul of template DNA. PCR products were then visualized via gel electrophoresis to confirm a band at ~600 bp (Raja et al., 2017). After confirmation of the product by PCR and gel electrophoresis, Sanger sequencing of PCR products was performed by Eurofins Genomics, LLC in Louisville, KY. Once these sequences were obtained, they were analyzed using the NCBI Basic Local Alignment Search Tool (BLAST), using default parameters, against all *Peronospora* sequences in the database (Altschul et al., 1990).

Once COX2 sequences were obtained, they were aligned with those of three outgroups, *Peronospora effusa* (KT944073.1), *Phythophthora lagoriona* (JF273084.1), and *Pythium ultimum* (AF196636.1) (GenBank) using MAFFT alignment software v7.305b with default settings (Katoh and Standley, 2003). The resulting alignment was then trimmed at nucleotide positions 31 and 631 using a Python script 'clip_alignment.py' (Anthony Westbrook, unpublished work) to remove overhang caused by aligning shorter sample sequences to longer outgroup sequences (Appendix IV, https://github.com/twestbrookunh/misc/tree/master/clipAlignment). Phylogenetic trees were then inferred and compared using Random Axelerated Maximum Likelihood (RAxML, v.8.2.11, default settings) and IQTree (v.1.6.2, default settings) bootstrapping models ultrafast bootstrap (UFBoot) and SH-aLRT test (Stamatakis, 2014; Hoang et al., 2018).

RESULTS

Disease susceptibility and symptom expression

Accessions BYU 803, BYU 803 F, *C. quinoa* var. Faro, 37P quinoa, and QQ065 quinoa had the highest mean downy mildew disease severity ($p = 0.0002$) (Table 3-2). Accessions BVM Appledore, BVM Rye, BVM USDA, and CAW-1(*C. album*) had the lowest downy mildew disease severity over the 2018 season ($p \leq 0.0001$).

Each accession reached peak disease severity on the September 15, 2018 evaluation date, with the second-highest peak occurring on August 17, 2018 (Figure 3-5). For all of the accessions, except for the BVM accessions, symptoms began as foliar chlorosis, and then sporulation signs appeared, and the disease escalated at the very end of the season to cause defoliation. Accessions with heavy sporulation, BYU 803, BYU 803 F, *C. quinoa* cv. Faro, and quinoa 37P had sporulation occurring on the bottom and top of the leaf. Mulch type had a significant effect on disease severity, in which beds without black plastic mulch had a significantly higher average disease severity ($p = 0.0092$). Statistical analyses did not reveal any significant interaction between the effects of accession and mulch type on disease severity ($p = 0.0988$). There was also significant variation of the symptom expression of downy mildew infected accessions. Quinoa and quinoa hybrid accessions (BYU 803, BYU 803F, 37P, QQ065, Rainbow, and Faro) had higher incidence of foliar pink discoloration as a result of downy mildew infection than CAW-1 or BVM accessions (Table 3-3).

In the field, initial downy mildew symptoms (chlorosis and pink discoloration) began appearing on multiple *Chenopodium* accessions on July 27, 2018 (Figure 3-5). *P. variabilis* has been reported to thrive under high humidity, high leaf wetness, and moderate temperatures

(Danielson and Lubeck, 2010). Over the 4-month growing season (June - September) the average air temperature was 19°C and there was an average of 12.7 cm of rain per month. There were approximately 155 and 318 hours of leaf wetness, and relative humidity above 90%, respectively (Figures 3-3 and 3-4).

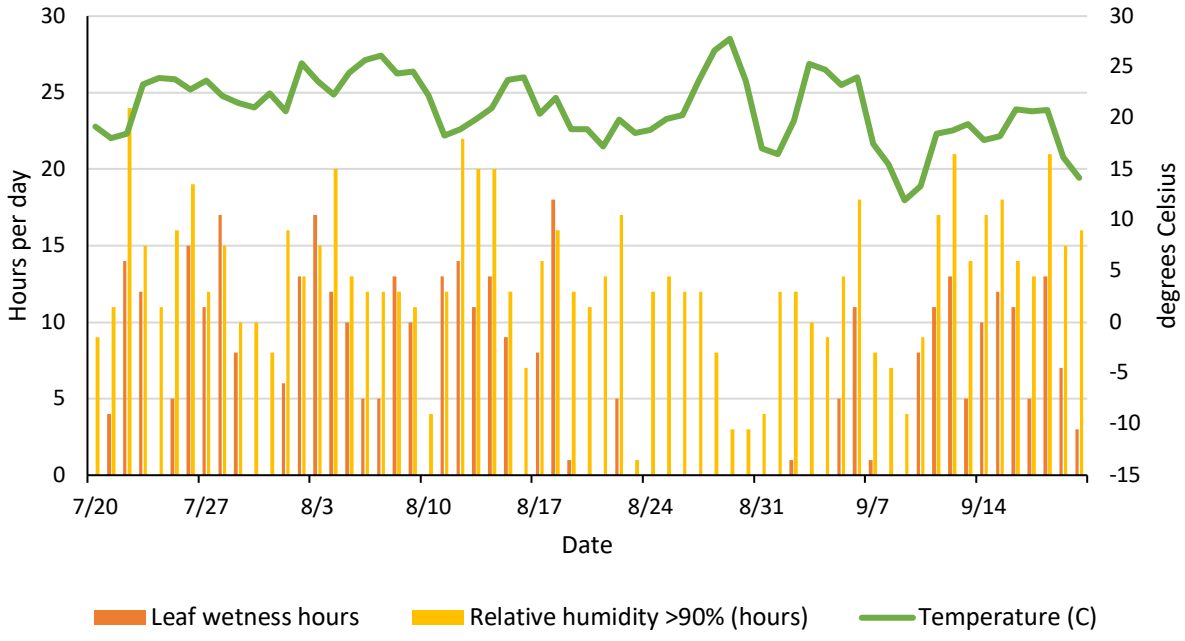

Figure 3-3. Combined bar chart and line graph of leaf wetness hours and relative humidity (bars, left axis), and average air temperature (line, right axis) from July 20, 2018 to September 16, 2018. Data obtained from the Network for Environment and Weather Applications.

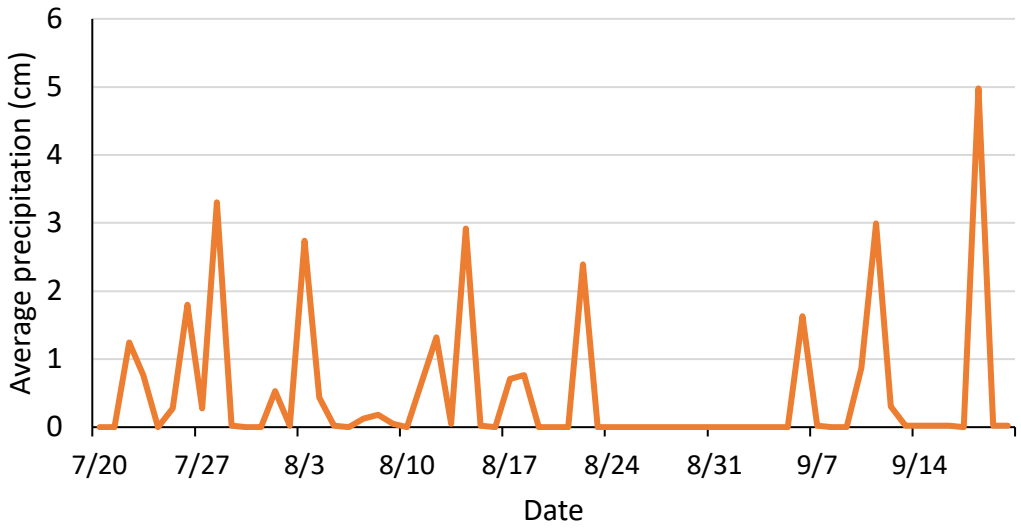

Figure 3-4. Average precipitation in cm per day from July 20, 2018 - September 16, 2018. Data obtained from the Network for Environment and Weather Applications.

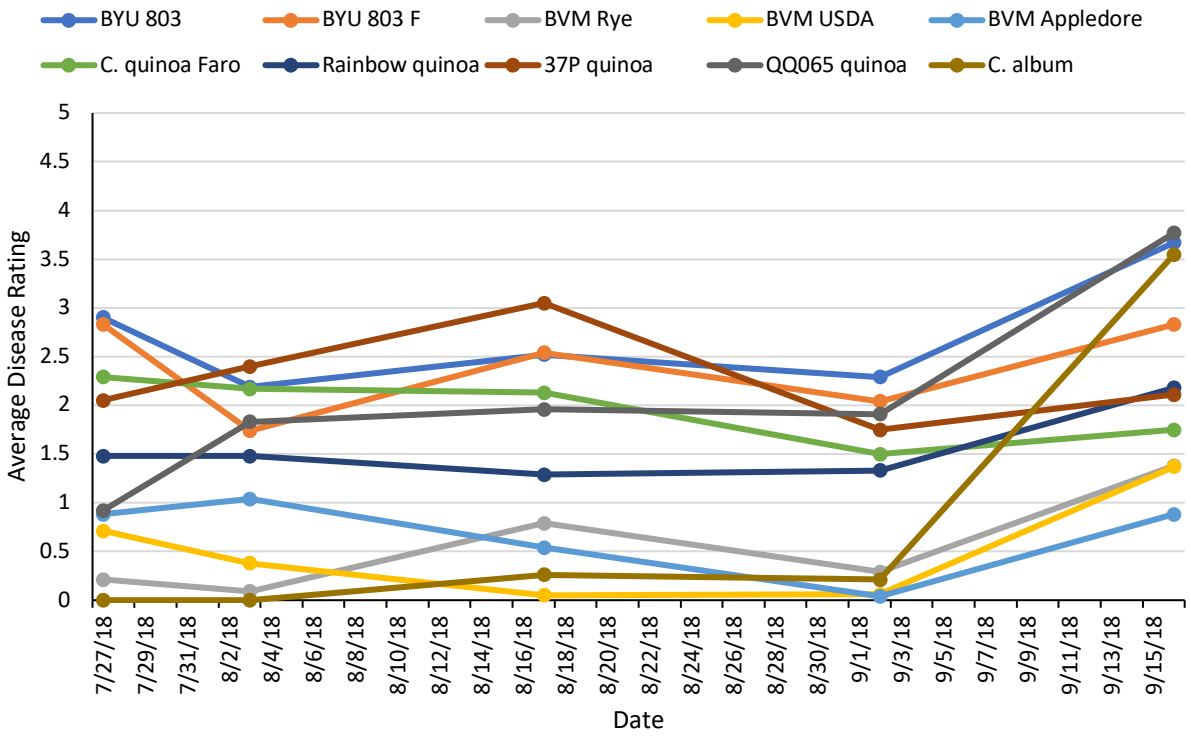

Figure 3-5. Downy mildew disease severity progression (on a 0-5 scale) on *Chenopodium* accessions over the 2018 growing season (July 27-September 16). Severity is the mean score of 24 plants in 6 replicate plots.

In addition to downy mildew, another disease was present during the field trial. Stem lesion symptoms were observed on multiple accessions in early September and continued to develop until the end of the field season (Figure 3-6). Symptoms observed began as small, 2-3 cm lesions with a black outer ring and white colored inner area with black pycnidia (asexual fruiting body) visible under a dissecting microscope. Lesions were seen on the primary stem and over time spread to the secondary branches. The causal agent of these stem lesion symptoms and disease severity data is discussed more in depth in Chapter Four. In order to assess *Chenopodium* susceptibility to all diseases present, disease severity scores were also recorded for stem lesion disease. Overall disease severity evaluations included disease ratings for symptoms present that could have been caused by other pathogens.

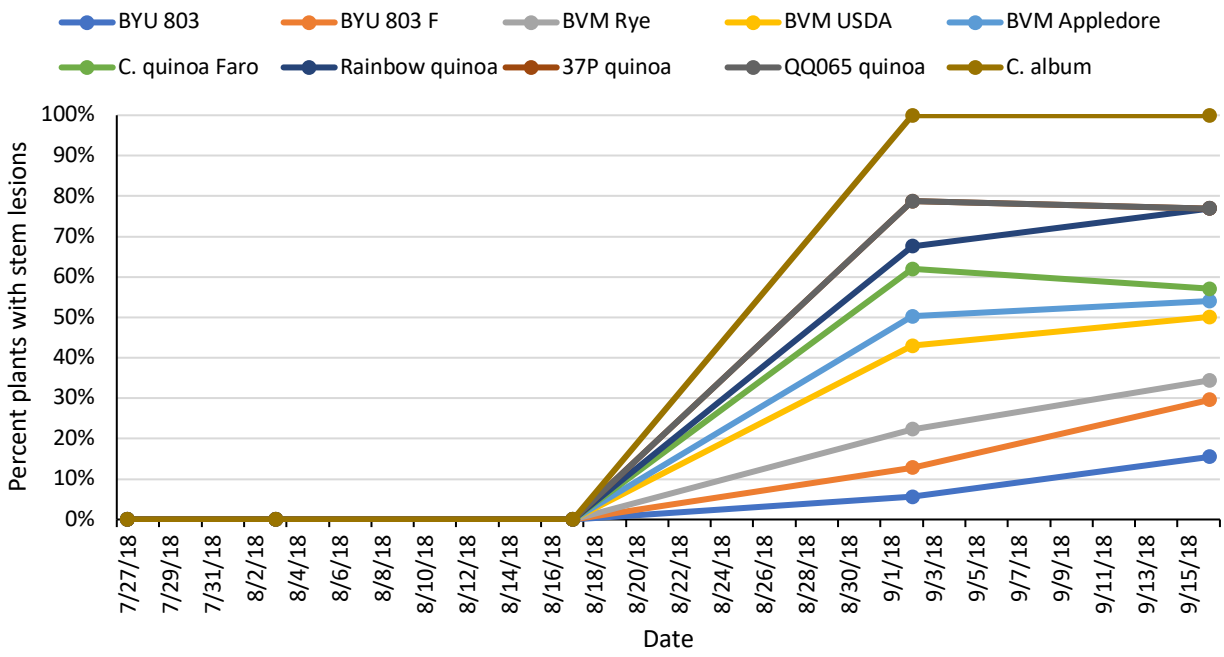

Figure 3-6. Mean stem lesion incidence (percent plants showing symptoms) on *Chenopodium* accessions over the 2018 growing season (July 27 – September 16).

Table 3-2. Mean downy mildew disease severity (using a 0-5 scale) per *Chenopodium* accession over time. Accessions not connected by the same letter within a column are significantly different at $\alpha = 0.05$ (n=24 per accession). Analyses were performed using repeat measures assessment using a residual covariance structure, analyzing disease severity over the entire Summer 2018 growing season.

	Downy mildew severity		
Treatment/accession	**Mean**		**Std Error**
BYU 803	2.702	a	0.152
QQ065	2.053	bc	0.145
BYU 803F	2.404	ab	0.144
37P	2.275	ab	0.155
Faro	1.966	bc	0.143
Rainbow	1.559	c	0.151
BVM Appledore	0.675	d	0.143
BVM Rye	0.554	d	0.143
BVM USDA	0.505	d	0.164
CAW-1	0.749	d	0.144

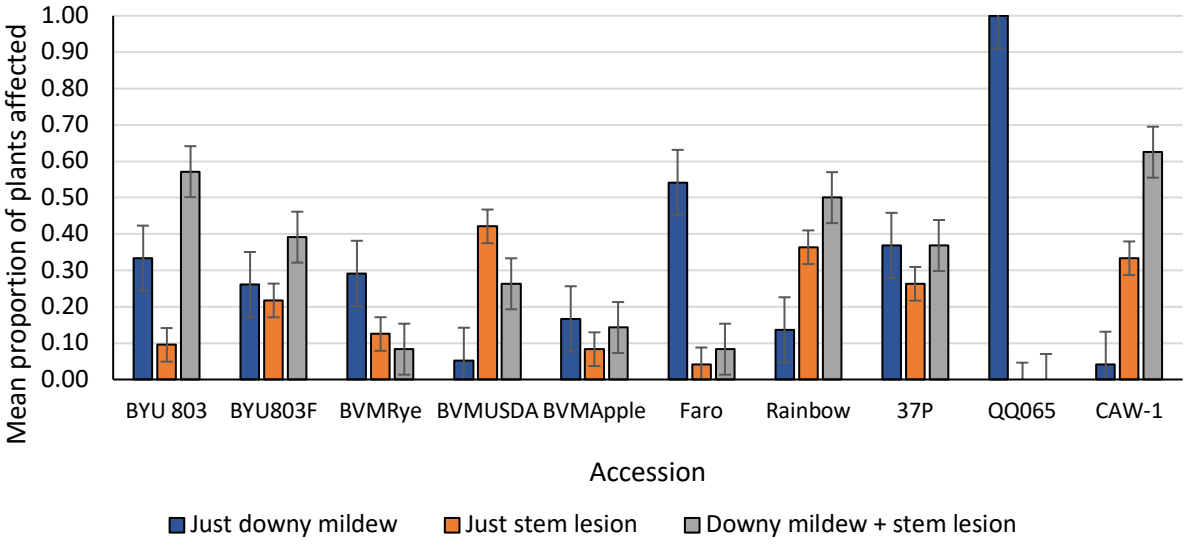

Figure 3-7. Mean proportion of plants affected by downy mildew (blue), stem lesions, (orange), and both diseases (gray). Bars represent standard error of the means.

Table 3-3. Mean foliar pink discoloration symptom incidence on July 27, 2018. Accessions not connected by the same letter are significantly different. (n=24 per accession)

	Pink spot incidence		
Treatment/accession	**Mean**		**Std Error**
BYU 803	32%	a	0.105
QQ065	12.5%	abc	0.069
BYU 803F	12.5%	abc	0.069
37P	0%	bc	0.000
Faro	16.7%	abc	0.077
Rainbow	0%	bc	0.000
BVM Appledore	29.1%	abc	0.095
BVM Rye	37.5%	a	0.101
BVM USDA	5.8%	abc	0.058
CAW-1	0%	c	0.000

Area under the disease progress curve

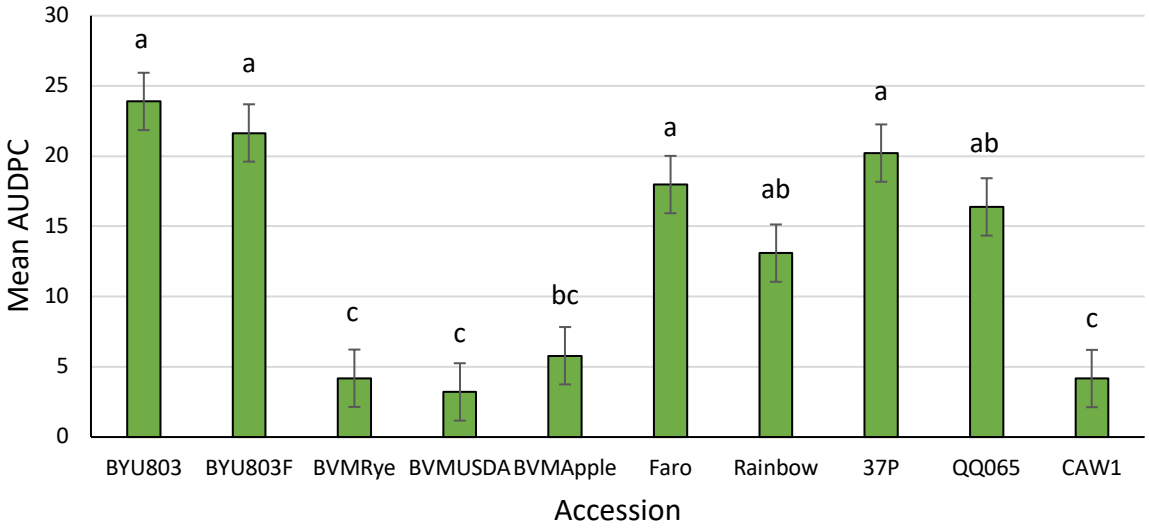

Figure 3-8. Mean downy mildew area under the disease progress curve (AUDPC) for each accession evaluated in Summer 2018. Error bars represent the standard error of the mean for each data set. Bars not connected by the same letter are statistically different ($p < 0.05$).

Phylogenetic analyses

The best supported tree (bootstrap ≥ 70%) can be seen in Figure 3-9, showing the relationships between different *P. variabilis* isolates collected from different *Chenopodium* species. The distance matrix shows very little sequence dissimilarity between the taxa (Figure 3-10). This tree shows no clear relationships among *P. variabilis* isolates from different *Chenopodium* species and does not support claims by other researchers that isolates are host species specific. However, isolates from only three *Chenopodium* species were included in this tree and none of these species were included in previous cross infection research.

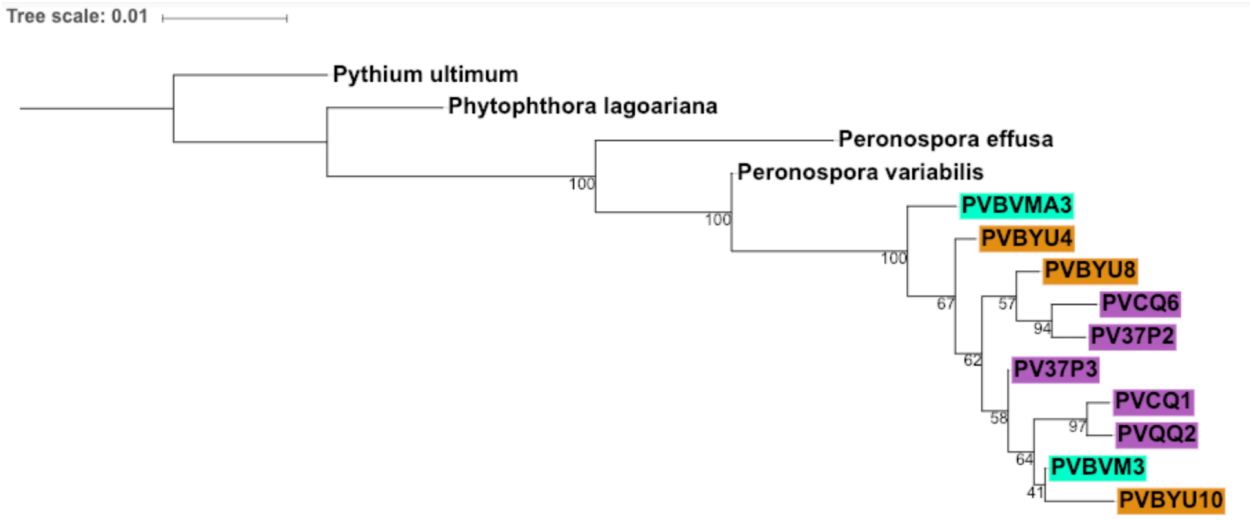

Figure 3-9. Inferred RAxML COX2 phylogenetic tree of collected *P. variabilis* isolates from different *Chenopodium* hosts: BVM Apple (blue), BYU 803 (orange), and quinoa (purple). Numbers located at each node are bootstrap values.

Isolate	Divergence												
AF196636 Pythium													
DQ365734_1 P. variabilis	0.091												
JF273084_1_Phytophthora_lagoariana	0.060	0.064											
KT944073_1 P. effusa	0.107	0.051	0.078										
PV37P2	0.096	0.006	0.070	0.049									
PV37P3	0.096	0.006	0.070	0.049	0.000								
PVBVM3	0.096	0.006	0.070	0.049	0.000	0.000							
PVBVMA3	0.091	0.000	0.064	0.051	0.006	0.006	0.006						
PVBYU10	0.096	0.006	0.070	0.049	0.000	0.000	0.000	0.006					
PVBYU4	0.096	0.006	0.070	0.049	0.000	0.000	0.000	0.006	0.000				
PVBYU8	0.096	0.006	0.070	0.049	0.000	0.000	0.000	0.006	0.000	0.000			
PVCQ1	0.096	0.006	0.070	0.049	0.000	0.000	0.000	0.006	0.000	0.000	0.000		
PVCQ6	0.096	0.006	0.070	0.049	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	
PVQQ2	0.096	0.006	0.070	0.049	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.000

Figure 3-10. Distance matrix of New Hampshire *P. variabilis* isolates COX2 sequences.

DISCUSSION

The purpose of this field trial was to observe and evaluate how different *Chenopodium* accessions react to infection in a natural setting, and to observe the timeline of downy mildew infection in the field. Disease severity data and AUDPC analyses indicate that there is significant differential resistance/susceptibility to the *P. variabilis* pathotype present in NH and other diseases among the accessions evaluated.

The *Chenopodium berlandieri* var. *macrocalycium* ecotypes from Rye Beach, NH and Appledore Island, ME had the lowest overall susceptibility to downy mildew and had the lowest disease incidence, suggesting that native wild species are tolerant and are a potential source of resistance genes to the *P. variabilis* isolates present in New Hampshire. It is also important to note that none of the accessions evaluated were completely resistant or immune, this continuous distribution of phenotypes suggests that quantitative/horizontal resistance may be responsible, with multiple genes contributing to disease resistance. The presence of horizontal resistance to quinoa downy mildew was also concluded by Kitz (2008) in a study of five quinoa breeding lines, with three lines (0654, Sayana, and NL6) showing evidence of possible quantitative resistance (Kitz, 2008). The results of this study also show that in addition to downy mildew, there are one or more other pathogens causing stem lesion symptoms that significantly affects *Chenopodium* species and that should be investigated in future studies (discussed further in Chapter Four).

Phylogenetic analyses did not support they hypothesis of host plant effecting intraspecific isolate relationships. *P. variabilis* isolates infecting *Chenopodium* species (BVM and a BVM x quinoa hybrid), are very similar to *P. variabilis* isolates infecting quinoa. These data are consistent with phylogenetic analyses performed by Testen et al. (2014) showing a regional

separation between South American and Pennsylvania isolates. These results also suggest that there are no host-species specific *P. variabilis* special forms, however these isolates may have differences in host-specificity that cannot be detected with the use of COX2 primers; this hypothesis may be the supported by the high sequence similarity shown between the taxa in Figure 3-10. Other loci within the genome (such as those connected to virulence) may be more informative for use in phylogenetic analyses. One limitation of the phylogenetic analyses performed is that isolates from only three different species were analyzed. It is possible that *P. variabilis* isolates are host species specific, but do not differ among the species included in this study, or that the accessions tested were not hosts to special forms present in the field. Further phylogenetic studies with finer resolution genetic markers or phylogenomic analyses as well as more North American sequence data would be beneficial in answering questions regarding *P. variabilis* population structure, evolution, and distribution in North America. Additionally, oospores have not been detected in New Hampshire *P. variabilis* samples to date (data not shown), suggesting this population is not sexually reproducing and undergoing very little genetic variation, which in turn affects the relationships among the isolates and potential for evolution within the population.

However, since this study included such a small sequence data set, these relationships should not be ruled out. In the future, more *P. variabilis* isolates will be collected from around New Hampshire to be added to the tree and increase the sample size, and hopefully the reliability and resolution of the inferred tree. In the sequence alignment and distance matrix (Figure 3-6), collected *P. variabilis* isolates can be seen to be extremely similar, with few single nucleotide polymorphisms contributing to the separation of sister taxa; it is expected that as more isolates are collected and sequenced, more information will be revealed about the relationships between

isolates. *P. variabilis* isolates will continue to be collected from *Chenopodium* species at Woodman Farm and in the Northern New England region to be isolated, sequenced, and added to this tree.

When performing field trials, many uncontrollable factors can arise. An issue that arose at the beginning of the summer was the difference in developmental ages between plants that were directly seeded versus transplanted, which can affect downy mildew severity and symptom expression. Another issue that arose was the presence of secondary pathogens. In a non-laboratory environment, there are many unknown microbial interactions happening with the host and its environment. Specifically, a stem lesion/stalk rot disease, also infecting *Chenopodium* plants was observed. The presence of other pathogens made it difficult to assess an accession's susceptibility to downy mildew specifically. There were some accessions (BVM Apple, BVM Rye, BVM USDA) that were tolerant to both diseases as well as accessions (CAW-1) that were tolerant to downy mildew but highly susceptible to stem lesions. The BVM USDA accession obtained from the USDA NPGS originated in Maine, suggesting that New England local ecotypes of BVM are the best source of tolerance to local diseases. Another complication in this study was poor germination rates in BYU 803 and BVM USDA accessions, which resulted in smaller sample sizes compared to the other accessions.

The results of this study support the hypothesis that there are different levels of resistance to the downy mildew pathogen within the genus *Chenopodium* (Table 3-2). The data also suggests the possibility of differential resistance to the stem lesion causal agent, an advantageous trait to possibly incorporate into future breeding efforts. This presence of differential resistance and identification of *Chenopodium* species tolerant to both downy mildew and stem lesions allows us to perform future studies targeted at these tolerant species using controlled inoculation

and genetic tests to further investigate the genetic basis behind resistance in *Chenopodium* species. Eventually, these tolerant species and identified resistance genes will be used in breeding programs aimed to develop disease tolerant quinoa varieties.

In the future, field analyses will be continued with more accessions to be evaluated. These field analyses also provide native downy mildew isolates to use in genetic, phylogenetic, and cross-inoculation studies. The next steps after confirming differential resistance in the field is to perform cross-inoculations among different accessions with different *P. variabilis* isolates in an environmentally controlled growth room and observing disease severity.

CHAPTER FOUR: INVESTIGATING THE CAUSAL AGENT OF A STEM LESION DISEASE PRESENT ON *CHENOPODIUM* SPP. AT WOODMAN FARM IN DURHAM, NH

INTRODUCTION

Chenopodium quinoa (quinoa) has been identified by UNH scientists as a key potential crop for New England agriculture because of its nutritive properties and environmental adaptability. The overall goal is to domesticate a wild relative of quinoa for commercial farming in New England, using quinoa as a model. However, there are many obstacles in achieving this goal; a significant obstacle being susceptibility to disease. Quinoa is susceptible to infection by several plant pathogens causing diseases such as downy mildew, damping-off, blight, leaf spot, and stalk rot (Danielsen et al., 2003). Downy mildew is the most destructive and well-documented disease on quinoa and has been reported in several countries. However, during field studies conducted at the UNH research farm in 2018, another disease was found causing significant damage to several *Chenopodium* spp. accessions (Figure 4-1). Symptoms seen at Woodman Farm included expanding stem lesions that coalesced and weakened the stem, contributing to issues of lodging. A disease that causes similar symptoms is brown stalk rot, caused by *Phoma exigua* var. *foveata* (Foister) Boerema, has been reported frequently on quinoa in the Andean highlands (Alandia et al., 1979). *P. exigua* var. *foveata* is a soil-borne pathogen that is commonly seen in areas with lower temperatures and high humidity (Danielsen et al, 2013). The brown stalk rot infection begins as small lesions on the upper third of the stalk that grow to cover more of the stalk and inflorescence, eventually leading to the bending and

breaking of the main stalk (Danielsen et al., 2003). A key sign of *P. exigua* var. *foveata* is also visible pycnidia within the lesions (Danielsen et al., 2003). This disease could be a significant limiting factor to New England farmers growing quinoa in the future and thus is important to consider in future disease resistance studies and breeding efforts.

The appearance of this new disease sheds light on the vast lack of knowledge of the various organisms causing disease on *Chenopodium* hosts in North America. The objective of this study was to identify the causal organism that was causing the stem lesion disease in our NH field plots, and test the hypothesis that lesions were caused by *Phoma exigua* var. *foveata*.

In this study we used morphological and molecular identification techniques to identify the causal agent of the unreported stem lesions disease recently observed in New England. The identification of causal agents of disease is important to implement effective disease control strategies and is a critical step to ensure reproducibility in plant pathology (Raja et al., 2017). This study included both molecular and morphological identification techniques, as morphological techniques alone may be misleading because of hybridization of species, convergent evolution, and differences in naming of sexual and asexual stages (Raja et al., 2017). In addition to these challenges, sexual structures are often used in morphological identification and isolates do not always sporulate in culture and conidia can be highly plastic within species (Raja et al., 2017).

Figure 4-1. Stem lesion symptoms observed on *C. quinoa* 37P in summer of 2018 at the Woodman Horticultural Research Farm in Durham, NH. Photo: H. Nolen.

METHODS

Collection and culture of candidate fungal isolates

Stem lesions were first observed within research plots at the Woodman Farm in Durham, NH on September 2, 2018 (Appendix VI). Diseased stems were collected in the research field from a *C. ficifolium* plant from an adjacent plot. In the lab, black pycnidia (asexual fruiting bodies produced by some ascomycetes) were observed inside the lesions under a dissecting microscope (Olympus SZ61) and were scraped off with a sterile dissecting needle and plated on potato dextrose agar (PDA). The cultures were allowed to grow at room temperature in the dark and six days later the hyphae emerging from the pycnidia were subcultured using a flame sterilized cork borer (5 mm diameter, Humboldt MFG Company) and plated on fresh PDA media.

Subcultures were performed a second time to obtain pure cultures of morphologically different isolates. Wet mount slides were used to look for the presence of spores in each isolate. For the sporulating isolates, single spore isolations were performed by flooding the petri dish with 10 ml sterile water and dislodging spores using a polypropylene cell spreader (Fisherbrand). The supernatant was poured into a small, sterile beaker and agitated with a spreader to mix. The mixed solution was filtered through two layers of sterile cheesecloth and the presence of spores was confirmed under the microscope at 40x objective (Olympus CX43). Fifty ul of the undiluted filtered spore suspension was plated onto 0.085% acidified PDA (APDA). Two tenfold dilutions were also prepared and aliquots of 50 ul were pipetted onto APDA plates. APDA was used to slow down the growth of the fungi to assist in isolation of colonies originating from a single germinating spore. Once colonies started growing, single colonies were subcultured using a 5 mm cork borer and plated on to fresh PDA.

For the non-sporulating isolate, the hyphal tip method was used to obtain a single isolate pure culture. Only the very edge of the colony was collected and subcultured. Once the colony started to grow, a hyphal tip was collected using a 5 mm cork borer and transferred to a new PDA plate. Two more rounds of subcultures were performed to ensure purity of the isolate.

Microscopic identification of the unknown fungal isolates

Hyphae were isolated from pure cultures of each isolate with a sterile dissecting needle and placed in reverse osmosis (RO) water on a glass slide. A cover slip was placed over the sample and microscopy was performed with an Olympus CX43 microscope at the 40X objective. Microscopy was performed to document morphological features that may aid in the identification of the unknown fungi, such as the presence of septate or non-septate hyphae or certain types of

sporulating structures. Olympus CellSens Entry software was used to record measurements of spores and sporangia at the 20X objective.

Molecular identification of the unknown fungal isolates

DNA extraction, PCR, and gel electrophoresis

DNA was extracted from the single spore fungal isolates using the cetyltrimethylammonium bromide (CTAB)-based technique as described by Torres et al. (1993). Cultures were flooded with 10 ml sterile tap water and scraped with a cell spreader. The supernatant was poured into a small beaker. The suspended hyphae and spores were pipetted into a 1.5 ml microcentrifuge tube and centrifuged at 14,000 RPM (Microfuge 18 Centrifuge, Beckman Coulter Diagnostics). The pellet removed from the microcentrifuge tube and was frozen in liquid nitrogen and crushed with a sterile mortar and pestle. DNA extractions were performed via the CTAB method as described in Chapter Two ("DNA extractions"). Once isolated, DNA was amplified by PCR according to the following thermocycler profile: initial denaturation step at 95°C for 2 min; followed by 30 cycles with denaturation at 95°C for 20 s, annealing at 55°C for 25 s, and elongation at 65°C for 2 min, with a final elongation step at 65°C for 10 min using an Eppendorf Mastercycler ep (Brinkmann Instruments). PCR was performed with forward and reverse internal transcribed spacer region (ITS) and large subunit (LSU) primer pairs described in Raja et al. (2017) (Table 4-1). The official barcoding region for fungal organisms is the internal transcribed spacer region (ITS), which is the fastest evolving region of the fungal genome and is often used to identify fungi to the species level (Raja et al., 2017). The large ribosomal subunit is considered to be a slower evolving region than the ITS and can be used to identify fungi to the genus level (Raja et al., 2017). These primers were used in combination to ensure a correct molecular identification. For these regions, 25-ul PCR reactions

were performed with 1X LongAmp *Taq* DNA Polymerase (New England Biolabs), 1X LongAmp *Taq* Reaction Buffer, 0.2 uM of each primer, 300 uM dNTP's, and 5 ul of template DNA. PCR products were visualized via 1% agarose gel electrophoresis to confirm bands at ~1200 bp (ITS primers) and ~600 bp (LSU primers).

Table 4-3. List of primers used in this study (Raja et al., 2017).

Primer name	Region	Sequence (5' to 3')
LR6	28s large ribosomal subunit	CGCCAGTTCTGCTTACC
LROR	28s large ribosomal subunit	ACCCGCTGAACTTAAGC
ITS4	5.8s internal transcribed spacer	TCCTCCGCTTATTGATATGC
ITS5	5.8s internal transcribed spacer	GGAAGTAAAAGTCGTAACAAGG

Sequencing and BLAST

PCR products were purified using Zymo DNA Clean and Concentrator Kit and were sequenced by Eurofins Genomics, LLC in Louisville, KY. Once sequences were obtained, they were analyzed using the National Center for Biotechnology Institute (NCBI) Basic Local Alignment Search Tool (BLASTn) and compared against all fungal and all oomycete sequences in the database (Altschul et al., 1990).

Pathogenicity of the fungal isolates

The pathogenicity of the single spore isolates was compared to fulfill Koch's postulates and identify the causal agent of the stem lesions. CAW-1, BVM Apple, *C. ficifolium*, and quinoa 37P plants were inoculated with the three different fungi. Plants were wounded by dusting leaves and stems with carborundum powder and then washing off with tap water. Both leaves and stems

were wounded for spray inoculations in order to determine if the candidate organism would cause the stem lesion symptoms or some other foliar symptoms. Inoculum of each isolate was prepared by flooding culture plates with 20 ml sterile tap water and scraping off hyphae and spores with a cell spreader. The supernatant was filtered with sterile cheesecloth and 10 ul Tween 20 (VWR) was added to each 50 ml suspension. Inoculation protocols differed among the fungal isolates. Three different species were identified via molecular analyses, one *Alternaria* species and two *Fusarium* species. For *Alternaria* isolates, plants were sprayed with Preval sprayers (Preval, Coal City, IL) and stems were painted with a spore suspension using a powder makeup brush (https://www.amazon.com/Matto-Bamboo-Makeup-Kabuki-Pieces/dp/B01JZAMNXM/ref=sr_1_21_s_it?s=beauty&ie=UTF8&qid=1547567677&sr=1-21&keywords=foundation+brush&refinements=p_36%3A1253950011). For *Fusarium* isolates, plants were either sprayed with inoculum or 10 ml of inoculum was pipetted directly into the soil. Treatments included: *Alternaria* spray, *Alternaria* paint, *Alternaria* spray + paint, *Fusarium* spray, *Fusarium* drench, and *Fusarium* spray + drench, with four control plants that were uninoculated. Plants were grown in 8 cm pots and kept in a growth room at 22°C and 74% humidity, with a 16-hour light/8-hour dark photoperiod following inoculation. This experiment was a randomized split plot design, with inoculant as the blocking factor and three replicate plants per treatment.

Disease assessment and data analysis

As described in Chapter 3, a field trial investigating disease resistance among *Chenopodium* accessions was performed during the 2018 summer growing season. The experiment consisted of a 2-factor randomized split plot design with 10 *Chenopodium* accessions (Table 3-1) and 2 mulching systems (black plastic or none) for a total of 20 treatments with three

replicate plots per treatment. A total of 240 individual plants were planted in six rows, with each row consisting of 10 subplots representing each accession composed of 4 plants of the same accession. While the study was focused on the incidence and severity of quinoa downy mildew, stem lesion disease appeared suddenly on September 2, 2018 (Appendix VI). Disease assessments for this new disease were included in data collection, with three subplots (12 plants total) contributing to an average stem lesion severity value. Stem lesion disease severity was analyzed based on the percent of the entire plant affected as well as a 0/1 (absent/present) scale and disease evaluations were performed biweekly. Presence/absence data were used to calculate an incidence value, in the manner depicted below. Stem lesion severity data were not initially fitting a normal distribution and were transformed using a square root transformation to fit a normal distribution. Transformed severity data were analyzed using a two-way analysis of variance (ANOVA) with repeated measures using JMP 14 software and an $\alpha = 0.05$. Post-hoc tests were done using mean separation Tukey Kramer tests.

$$Stem\ lesion\ incidence = \frac{number\ of\ plants\ in\ subplot\ with\ stem\ lesions}{total\ number\ of\ plants\ in\ subplot} * 100$$

RESULTS

Field conditions and symptoms observed

In the field, 2-3 cm lesions were observed on multiple *Chenopodium* accessions. These lesions had a black outer ring and a white colored inner area (Figure 4-1), with black pycnidia visible under a dissecting microscope. Lesions were seen on the primary stem of the plants as

well as secondary branches. Over the 4-month growing season (June - September) the average air temperature was 19°C and there was an average of 12.7 cm of rain per month. There were approximately 155 and 318 hours of leaf wetness, and relative humidity above 90%, respectively (Figures 3-3 and 3-4).

Isolation and identification of unknown isolates

PCR and BLAST analyses

Four morphologically distinct fungi were isolated from the collected plant material (SL-Fic-A, B, C, and D). Based on PCR and BLAST analyses, three potential causal organisms for the stem lesion disease were found: *Alternaria alternata*, *Fusarium sporotrichioides*, and *Fusarium equiseti* (Table 4-2).

Table 4-4. BLAST results of the ITS sequences of the four fungi isolated from *C. ficifolium* stem lesions.

Isolate ID	BLAST Result	E-Score	Per. Identity	Accession
SL-Fic-A	*Alternaria alternata*	0.0	99.63%	MH886523.1
SL-Fic-B	*Fusarium sporotrichioides*	0.0	99.62%	MG274314.1
SL-Fic-C	*Fusarium equiseti*	0.0	99.62%	MK168567.1
SL-Fic-D	*Fusarium equiseti*	0.0	99.62%	KX576658.1

Macroscopic identification

Cultures of SL-Fic-A were slower growing relative to the other isolates and had black/gray mycelia with the newest growth appearing as a white ring around the colony. SL-Fic-B, C, and D were all a pale-yellow color, with C and D having floccose, aerial mycelia. SL-Fic-C and D cultures were slightly darker in color than those of SL-Fic-B. SL-Fic-C and D were initially kept as distinct samples because of a slight color difference in hyphae, however

BLASTn analyses suggests they were the same species. SL-Fic-B also had aerial mycelia, and hyphae formed in string-link clusters, called sporodachia (Domsch et al., 1993).

Microscopic identification

The isolate (SL-Fic-A) identified as *A. alternata* did not sporulate when grown on PDA media and had septate hyphae, branching at ~90°, with approximately 20 um increments between septa (Figure 4-2A). The two *Fusarium* species isolates produced thin-walled, globose chlamydospores (10 to 22 um diameter) in culture and had septate hyphae (Figures 4-2B, C, 4-3).

Figure 4-2. Microscopic images taken at 20X objective of (A) *Alternaria alternata*, (B) *Fusarium sporotrichioides*, and (C) *Fusarium equiseti*.

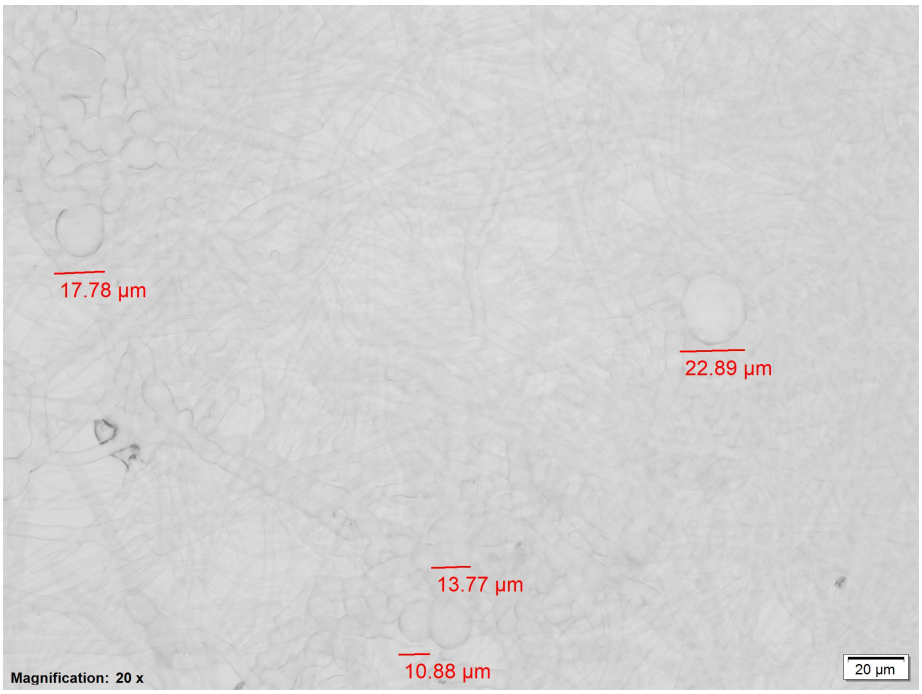

Figure 4-3. Microscopic image taken at the 20X objective of *F. equiseti* spores and hyphae.

Pathogenicity of the isolates

Thus far, inoculations of CAW-1, BVM Apple, *C. ficifolium*, and quinoa 37P plants with the fungal isolates have not resulted in symptom expression.

Disease severity analysis

Significant differences in susceptibility to stem lesions were seen among the different *Chenopodium* accessions planted in the field. Quinoa accession QQ065 (PI 614880) and *C. berlandieri* var. *macrocalycium* obtained from the USDA (BVM USDA, PI 666279) had the lowest disease incidence (Figure 4-4).

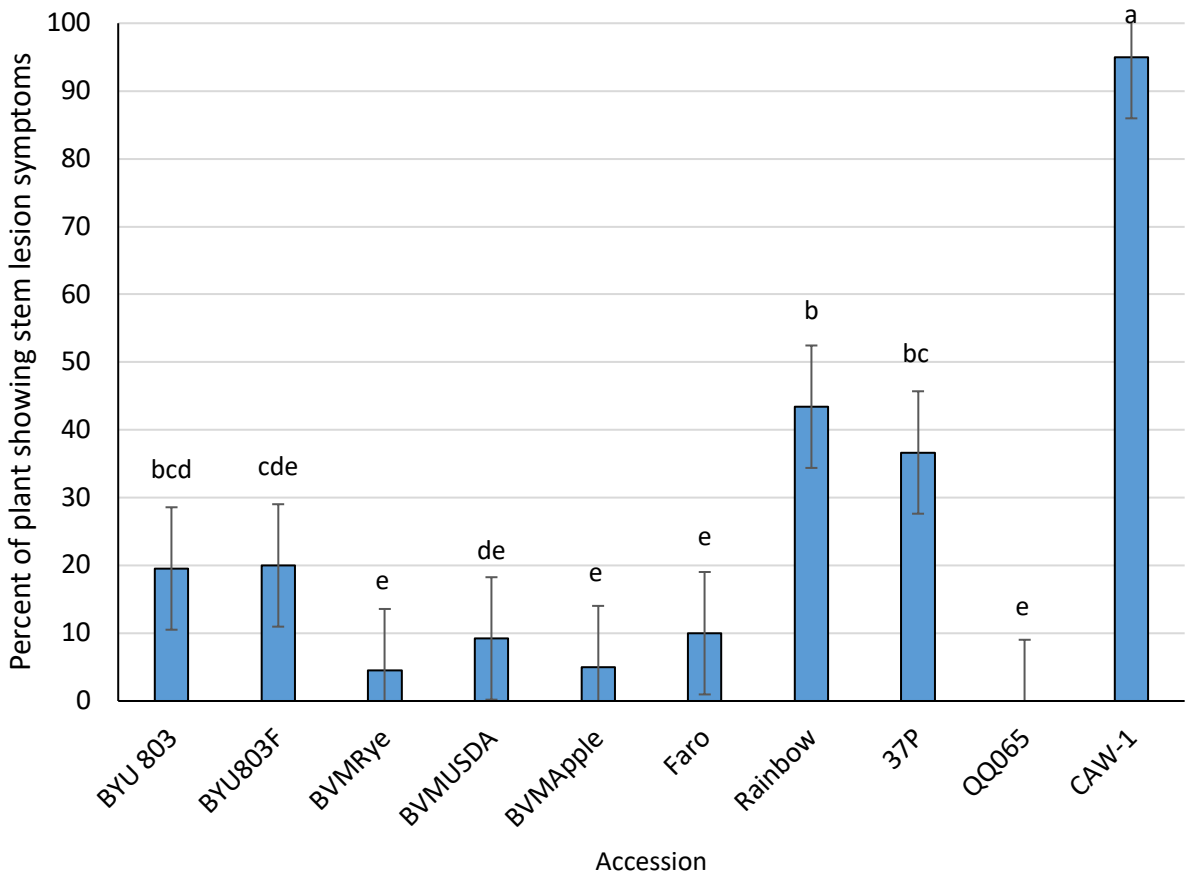

Figure 4-4. Mean percent of plant showing stem lesion symptoms at the end of the 2018 growing season for each accession. Bars represent the standard error of the mean. Bars not connected by the same letter are statistically significant ($p < 0.05$).

DISCUSSION

In order for quinoa to be productively grown in New England disease management strategies are needed to reduced losses to disease. To develop effective strategies against disease it is imperative to document the potential pathogens present in New England, determine quinoa cultivar susceptibility to these diseases, and identify the causal organisms of those diseases. While downy mildew is the most significant disease affecting the *Chenopodium* genus

(Danielsen et al., 2003), the stem lesion disease described above was present in moderate to high severity on many *Chenopodium* accessions during the summer 2018 growing season. Interestingly, comparing Figures 3-7 and 4-4, incidence and severity of the stem lesion disease do not seem to be directly related. The CAW-1 accession had the highest incidence (Figure 3-7), and the highest affected area, however, BYU 803 had higher incidence of the stem lesion disease with relatively low affected area (an average of 20% of the plants showing symptoms) (Figure 4-4). These differences may suggest multiple genes at contributing to the tolerance of the stem lesion disease. The causal agent of the disease is still unknown, but this study has identified three potential causal organisms: *Alternaria alternata*, *Fusarium sporotrichioides*, and *Fusarium equiseti*.

A. alternata has been reported on quinoa in Brazil and on *C. album* in The Netherlands (Mendes et al., 1998; Woudenberg et al., 2017). *F. equiseti* has also been reported on quinoa in Brazil and on *C. album* in Canada (Conners, 1967). Both *A. alternata* and *F. equiseti* have also been reported on an unidentified *Chenopodium* species in Kenya (Caretta et al., 1999).

These reports of *A. alternata* and *F. equiseti* around the world suggest that these causal organisms are ubiquitous on *Chenopodium* species across different geographical regions. However, according to information available in the National Fungal Database (Farr and Rossman, 2019). *F. sporotrichioides* has not been reported on any *Chenopodium* species throughout the world, suggesting this is not a common, cosmopolitan disease-causing organism, and that the form that infects *Chenopodium* may be confined to North America or the northern New England area.

Thus far, artificial inoculations have not resulted in development of similar stem lesions as seen in the field or any other symptoms. It is hypothesized that these inoculations were

unsuccessful due to the environmental conditions of the growth room they were inoculated in; while the humidity in the growth room was set at 74%, the plant tissue was not able to stay consistently wet. A damp environment and wet leaves are a critical component for the pathogen to be able to penetrate the leaf surface and cause infection. It is shown that in the weeks prior to initial symptom expression there were higher levels of precipitation, relative humidity, and leaf wetness hours (Figures 4-2, 4-3). These environmental conditions may have contributed to the initiation of the infection cycle of the unknown organism causing the disease and provided optimum conditions for the pathogen.

However, there are other possible explanations for the unsuccessful inoculations. It may be that none of these isolated organisms were the actual causal organism of the stem lesion disease and were just secondary pathogens that invaded plant material after initial infection. This explanation may be the case especially in regard to *F. sporotrichioides*, since it has never been documented as actually causing disease on *Chenopodium* species. These inoculations were repeated in a greenhouse setting in humid chambers with misters to keep the plants wet and still yielded no symptoms, suggesting that these isolated fungi were secondary pathogens that invaded host tissue after the true causal organism weakened the plant's defenses. Another possible explanation for the unsuccessful inoculations is that no treatment using mixed inoculum was tested. All of the fungi were initially isolated from a mixed culture, so it is possible the symptoms are a result of a synergistic relationship among multiple fungi. It is also noted in previous studies that *Phoma exigua* var. *foveata* causes a stalk rot disease on quinoa (Danielsen et al., 2003), however there are no images of infected plants from these reports, so we are unable to compare the symptomology of this reported stalk rot disease to the stem lesions observed in the field. If *P. exigua* var. *foveata* was present in the samples, it is expected that pale to yellow

brown, cylindrical to oval spores, similar to a closely related species *P. exigua* var. *exigua* would be present in wet mount samples observed under the microscope (Bardas et al., 2008).

Future studies

This study has not reached any definitive conclusions and thus future research is imperative in identifying what organism (or organisms) is causing this stem lesion disease. New isolates will be obtained during the summer 2019 growing season as soon as the symptoms appear in the field to attempt to obtain isolates before secondary organisms can invade the plant tissue. These isolates will be then subjected to the same pipeline described above, also including mixed isolate treatments to hopefully identify the specific species causing this stem lesion disease.

CHAPTER FIVE: DISCUSSION AND FUTURE DIRECTIONS

CONCLUSIONS

Chenopodium quinoa is a potential new specialty crop for New England agriculture; however, despite its many desirable agronomic characteristics, traits like lodging and disease susceptibility hinder the commercial potential of the crop. Researchers at the University of New Hampshire (UNH) are currently investigating the potential of "re-domesticating" quinoa in New England by using New England native *Chenopodium* relatives as a source of favorable genetic traits. *Peronospora variabilis*, the causal agent of quinoa downy mildew, is a detrimental pathogen of quinoa and, like many other downy mildews, is very difficult to control. Its spores can be spread by wind, rain, irrigation, farm equipment, human activity, and are able to travel great distances. Its sexual spores are able to overwinter in soil and infect the next season's crops, forcing quinoa farmers to either perform crop rotation or regular fungicide applications, which can be both labor and cost intensive. Utilizing genetically resistant crops has many advantages in agriculture. The use of resistant cultivars can reduce dependence on fungicides and chemical treatments, which can in turn help mitigate potential negative side effects of fungicides on soil microbial activity, air, and water quality. In this research, potential sources of disease resistance within the *Chenopodium* genus were identified. This project worked to investigate wild/weedy locally adapted *Chenopodium* species to incorporate in future quinoa breeding programs to develop disease resistant quinoa cultivars for crop production in New England.

The presence of *P. variabilis* in North America poses a significant obstacle to commercial production of quinoa in New England. The growing season from June to late

September provides a highly favorable climate (warm temperatures and high humidity) for the pathogen to grow and spread. Disease symptoms typical of downy mildew were first observed on quinoa in Durham, NH at the UNH Woodman Farm during the summer of 2016 and *P. variabilis* was confirmed by molecular identification in 2017. It is still unknown however, whether the pathogen was introduced on infected seed or if the pathogen was already prevalent in the region. *Chenopodium* spp. are native to the region and can be found throughout the state of New Hampshire and the northern New England area, suggesting that *P. variabilis* may have been prevalent in the region for some time, infecting these native weedy species. This study aimed to investigate the host-specificity of isolate *P. variabilis* strains from natural infection, the severity and spread of disease caused by this pathogen in farm setting and develop protocols for the efficient confirmation of *P. variabilis* in the field from seed and leaf tissue.

A molecular confirmation protocol has been developed for *P. variabilis* from seed and leaf tissue and field trial results support the hypothesis that there is differential resistance among *Chenopodium* species to disease. In the field trial (Chapter 3), there were significant differences in downy mildew severity, stem lesion incidence, and overall disease severity among the 10 *Chenopodium* accessions planted (Table 3-1). In this study, accessions were evaluated using the disease severity measurement, rather than an all or nothing susceptible or resistant phenotype. The finding of differences in degree of infection suggests that *Chenopodium* resistance may be quantitative and reliant on multiple genes within the host genome. Quantitative resistance is typically seen as a continuous phenotype and is controlled by multiple genes, versus qualitative resistance is expressed as an all or nothing, resistant or susceptible phenotype and is controlled by a single gene (Poland et al., 2008). Currently, there are very few genomic data and annotated sequences for either the host or the pathogen, and more studies are required to identify genes

conferring quantitative or qualitative resistance genes in the host and pathogenicity factors in the pathogen to understand this pathosystem in greater detail.

P. variabilis is a heterothallic organism; it has mating types (P1 and P2, Danielsen, 2001) that reside in different individuals and require individuals of two compatible mating types for sexual reproduction. To date, oospores have not been detected in material collected from New Hampshire, suggesting only one of the compatible mating types is present in the region. A greater sample size is needed to more conclusively determine which mating types are present in New England and identify the risk of sexual recombination in local *P. variabilis* populations. The lack of sexually compatible mating types may be due to the way the pathogen was introduced to the area. If *P. variabilis* was introduced by planting infected *Chenopodium* seed from South America or another region, different mating types may not have been present within the seed coat to sexually reproduce and proliferate throughout the region.

Phylogenetic analyses (Chapter 3) investigating *P. variabilis* isolates from different *Chenopodium* species did not support the initial hypothesis that there are genetic distinctions among isolates infecting different *Chenopodium* hosts. Further phylogenetic analyses, including New Hampshire isolates obtained in this study and COX2 sequences from the NCBI database reveal relationships dependent on geographical location of the isolate (Figure 5-1). A maximum likelihood tree inferred using IQTree ultrafast bootstrap interestingly shows a tree with three distinct clades: isolates from Pennsylvania (red) (Testen et al., 2014), isolates from New Hampshire (purple), and isolates from South American (yellow, orange) (Testen et al., 2014; Goker et al., 2007; Choi et al., 2015; Zhou and Bai, 2017; Jing, 2015). Interestingly, this highly supported tree (bootstrap ≥ 70%) shows that New Hampshire *P. variabilis* isolates have a greater affinity for South American isolates than the other North American isolates in the National

Center of Biotechnology Information (NCBI) database. This unexpected result may be due to the lack of North American *P. variabilis* sequence data available in the NCBI database. While certain *Chenopodium* species such as *C. album* (lambsquarters) are highly prevalent native weeds throughout the United States, there are relatively few North American institutions researching the plant or pathogen. The addition of geographically diverse isolates from North America may provide a more robust data set and reveal different relationships.

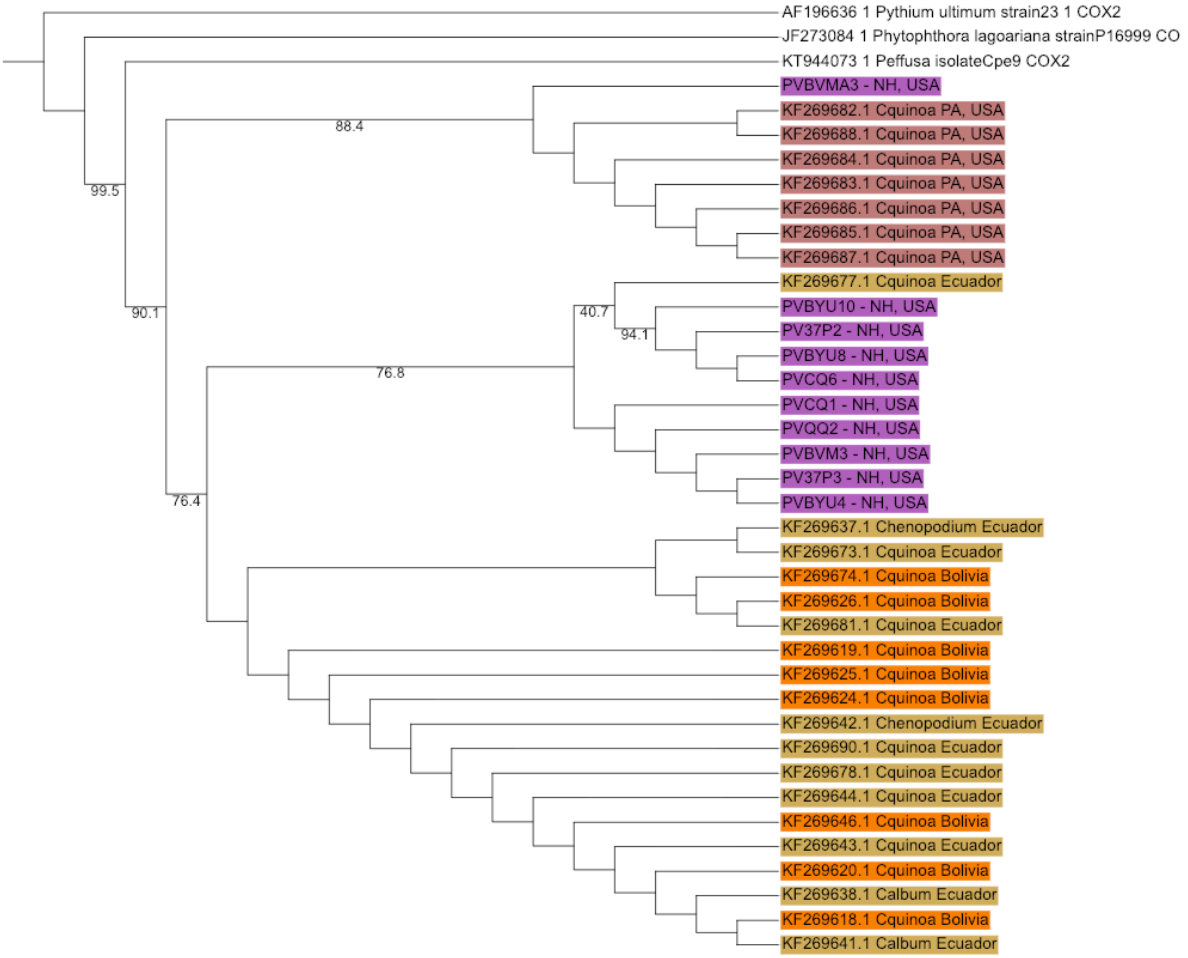

Figure 5-1. Inferred IQTree phylogenetic tree of New Hampshire collected *P. variabilis* isolates (purple), Pennsylvania isolates (red; Testen et al., 2014), and South American isolates (yellow, orange; Testen et al., 2014; Goker et al., 2007; Choi et al., 2015; Zhou and Bai, 2017; Jing, 2015). Numbers located at each node are bootstrap values.

As described in Chapter Four, a second disease was observed on *Chenopodium* spp. Differential susceptibility was observed among the *Chenopodium* species to this unidentified disease (Figures 4-1, 4-2). Currently, there are three possible candidate causal organisms responsible for the stem lesion disease: *Fusarium sporotrichiodes*, *Fusarium equiseti*, or *Alternaria alternata*. Since controlled inoculations did not result in stem lesion symptoms on *Chenopodium* seedlings, Koch's postulates could not be satisfied, and the causal agent could not be identified. Since samples were collected later in the growing season in September, it is possible that a secondary pathogen was isolated instead of the actual organism that caused this disease. However, it is also possible that one of these species is the causal organism and inoculations were not successful due to the environment in which the inoculations were performed. Thus far in these different studies, inoculations have not been successful with any pathogen (these candidate species or *P. variabilis*), suggesting that inoculations have not been performed in the optimal environment for infection. It is thought that conditions have been too dry, and leaves have not been consistently moist, allowing for the pathogen to travel and penetrate leaf and stem tissue.

Overall, this study has provided evidence of differential susceptibility and mulit-gene resistance to downy mildew within the *Chenopodium* genus, reports a potentially problematic stem lesion disease present in New Hampshire, and raises questions on the evolution and relationships among North American *P. variabilis* isolates. The *Chenopodium* accessions found to be the least susceptible to disease (*C. berlandieri* var. *macrocalycium* species from Rye Beach, NH and Appledore Island, ME) should be the focal point of future studies to identify candidate resistance genes for breeding new disease tolerant cultivars. Additionally, the reported stem lesion disease has the potential to hinder quinoa production in New Hampshire and more

information is needed about this disease and its causal organism to develop effective management strategies.

What is still unknown?

There are many aspects of the quinoa downy mildew system that are still unknown. The inferred tree (Figure 5-1) shows an unusual relationship between New Hampshire and South American isolates. It was expected for New Hampshire and Pennsylvania isolates to have higher likeness to one another due to their geographic proximity, however, the tree shows that New Hampshire isolates actually have a greater likeness for South American *P. variabilis* isolates. The relationships depicted in this tree raise the question of how *P. variabilis* was introduced into the United States. Did this pathogen migrate from South America, or is there a distinct *P. variabilis* isolate that was already present in North America on weedy *Chenopodium* species? Answering this question would probably require a substantially larger sequence data set of isolates from North America, which is unavailable at this time due to the very few North American researchers studying quinoa and its diseases.

At a genomic scale, there is little known about *P. variabilis*. Currently, the genome or transcriptome has not been sequenced or assembled. Because of this, quinoa downy mildew researchers have little insight into how many pathogenicity factors are involved in the infection process, and a lack of knowledge of how complex this disease system may be. There are also many aspects of the *P. variabilis* populations New England and even North America that are still unknown. The inferred tree (Figure 5-1) shows two clades of New Hampshire isolates, however what is causing this distinction, or if it will hold when more sequence data are added, is unknown. The clades are not separated by host plant, so it is unknown what exactly is causing this separation. There is also little known of the distribution and population structure of *P.*

variabilis throughout North America. *C. album* is widely distributed throughout the United States; thus, it can be assumed that *P. variabilis* infecting *C. album* is also present, however, there is little documentation of the pathogen throughout the country, leading to significant gaps in knowledge.

The genetic composition of the *P. variabilis* population is widely unknown. It is believed there is a great diversity of the pathogen population because the host genus has a high level of plasticity and diversity (Thines and Choi, 2016). The sexual states of *P. variabilis* are present in many areas of quinoa cultivation, allowing for recombination and increased genetic diversity. The geographic distribution of the pathogen also indicates higher probability of new mating types evolving, leading to the increase of genetic recombination and variation, thus introducing new pathogenic types. It is currently unknown if the genetic composition of *P. variabilis* in different populations is different, this lack of knowledge provides a risk of developing quinoa varieties resistant in certain locations but susceptible in others. We can't necessarily take what works for one location and apply it to another, but protocols used to determine a solution can be applied in different locations to reach appropriate solutions for those areas.

In regard to the host plant, it is still unknown what genes are conferring resistance to downy mildew in *Chenopodium*. Some candidate resistance genes are listed in NCBI, but there are no definitive studies confirming or denying these candidate genes. This study supports the hypothesis that there is differential resistance in the genus, but we still do not know the underlying genetic causes of this resistance.

Future studies

As described, many aspects of the quinoa downy mildew system are still unknown. Future studies should include controlled downy mildew inoculations in a lab or greenhouse study in order to further evaluate differential disease resistance in the genus. While informative, field trials have many uncontrollable factors that can affect the plant-microbe system. Because of this, it is imperative to include controlled inoculation studies to test the results obtained in the field trial. Future studies will also work to sequence and assemble the *P. variabilis* genome and transcriptome. Once these data sets are assembled, they will be mined for effector sequences, typically involved in pathogenicity factors, containing conserved sequences. In addition to searching for genes involved in pathogenicity, the genetic basis of mating types in this organism is also of interest. One future focus is to sequence isolates identified as different mating types to determine the loci contributing to distinct mating types. This work would provide critical knowledge of the genomics of the pathogen serve as preliminary work to other bioinformatic and genomic projects.

REFERENCES

Abugoch James, L.E. 2009. *Quinoa (Chenopodium quinoa Willd.): composition, chemistry, nutritional and functional properties.* Adv. Food Nutr. Res 58: 1-31.

Aegerter, B.J., Nunez, J.J., and Davis, R.M. 2002. *Detection and management of downy mildew in rose rootstock.* Plant Dis. 86: 1363-1368.

Ahamed, N.T., Singhal, R.S., Kulkarni, P.R., Pal, M. 1996. *Physicochemical and functional properties of Chenopodium quinoa starch.* Carbohydr. Polym. 31: 99-103.

Ahmad, S., Iqbal, S.H., and Khalid, A.N. 1997. *Fungi of Pakistan*. Sultan Ahmad Mycological Society of Pakistan, 248 pages.

Alandia, S., Otazu, V., Salas, B. 1979. Enfermedades. In: Tapia, M., Gandarillas, H., Alandia, S., Cardozo, A., Mujica, A., Ortiz, R., Otazu, V., Rea, J., Salas, B., Sanabria, E. eds. *Quinua y Kaniwa*. Bogota Colombia: IICA, pp. 137-148.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. 1990. *Basic local alignment search tool.* J. Mol. Biol. 215: 403-410. PubMed.

Aragon, L. and Gutierrez, W. 1992. *El mildiu en cuatro especies de Chenopodium.* Fitopatologia 27: 104-109.

Baiswar, P., Chandra, S., Kumar, R., and Ngachan, S.V. 2010. *Peronospora variabilis on Chenopodium murale in India*. Australasian Plant Disease Notes 5: 45-47.

Barboza, J., Ames, T., Jacobsen, S. E. 2000. *Reduccion de la germinacion y muerte de plantas de Chenopodium quinoa causadas por Rhizoctonia y Fusarium*. Memorias del Primer Taller Internacional sobre Quinoa., May 10-14, 1999, Lima, Peru in preparation La Molina: Centro Internacional de la Papa y Universidad Nacional Agraria.

Bardas, G.A., Tziros, G.T., and Tzavella-Klonari, K. 2008. *First report of Ascochyta leaf spot caused by Phoma exigua var. exigua on common bean in Greece.* Plant Disease 92(4).

Beckman, P.M., Finch, H.C. 1980. *Seed rot and damping-off of Chenopodium quinoa caused by Sclerotium rolfsii*. Plant Disease. 64:497-498.

Belbahri, L., Calmin, G., Pawlowski, J., and Lefort, F. 2005. *Phylogenetic analysis and real-time PCR detection of a presumably undescribed Peronospora species on sweet basil and sage*. Mycol. Res. 109:1276-1287.

Ben-Naim, Y., Falach, L., and Cohen, Y. 2018. *Transfer of downy mildew resistance from wild basil (Ocimum americanum) to sweet basil (O. basilicum).* Phytopathology 108: 114-123.

Bhargava, A., Shukla, S., and Ohri, D. 2006. *Chenopodium quinoa – An Indian perspective.* Industrial Crops and Products 23: 73-87.

Bininda-Edmonds, O.R.P. 2010. *SeqConverter.pl v1.2 Program distributed by the author.* AG Systematik and Evolutionsbiologie, IBU – Fakaultat V, Carl von Ossietzky Universitat Oldenburg.

Boerema, G.H., De Gruyter, J., Noordeloos, M.E., and Hamers, M.E.C. 2004. *Phoma identification manual: differentiation of specific and infra-specific taxa in culture.* CABI Publishing, 470 pages.

Botella, Miguel A., Parker, Jane E., Frost, Louise N., Bittner-Eddy, Peter D., Beynon, Jim L., Daniels, Michael J., Holub, Eric B., and Jones, Jonathan D.G. 1998. *Three genes of Arabidopsis RPP1 complex resistance locus recognize distinct Peronospora parasitica avirulence determinants.* The Plant Cell 10: 1847-1860.

Bourret, T., Choudhury, R., Mehl, H., Blomquist, C., McRoberts, N., and Rizzao, D. 2018. *Multiple origins of downy mildews and mito-nuclear discordance within the paraphyletic genus Phytophthora.* PLoS ONE 13: 1-23.

Brouwer, M., Lievens, B., Van Hemelrijck, W., Van den Ackerveken, G., Cammue, B.P.A., and Thomma, B.P.H.J. 2003. *Quantification of disease progression of several microbial pathogens on Arabidopsis thaliana using real-time fluorescence PCR.* FEMS Microbiol. Lett. 228:241-248.

Byford, W.J. 1967. *Host specialization of Peronospora farinosa on Beta, Spinacia, and Chenopodium.* Trans. Br. Mycol. Soc 50 (4):603-607.

Caretta, G., Piontelli, E., Picco, A.M., and Del Frate, G. 1999. *Some filamentous fungi on grassland vegetation from Kenya.* Mycopathologia 145: 155-169.

Chen, W., Delmotte, F., Richard-Cervera, S., Douence, C.G., and Corio-Costet, M.F. 2007. *At least two origins of fungicide resistance in grapevine downy mildew population.* Applied and Environmental Microbiology 73: 5162-5172.

Choi, Y.J., Denchev, C.M., and Shin, H.D. 2008. *Morphological and Molecular Analyses Support the Existence of Host-specific Peronospora Species Infecting Chenopodium.* Mycopathologia 165: 155-164.

Choi, Y.J., Danielsen, S., Lubeck, M., Hong, S.B., Delhey, R., and Shin, H.D**.** 2010. *Morphological and molecular characterization of the causal agent of downy mildew on quinoa* (*Chenopodium quinoa*). Mycopathologia 169: 403-412.

Choi, Y.J., Choi, I.Y., Kim, J.S., and Shin, H.D. 2014. *First report of quinoa downy mildew caused by Peronospora variabilis in Republic of Korea*. Pl. Dis. 98(7): 1003.

Choi, Y.J., Beakes, G., Glockling, S., Kruse, J., Nam, B., Nigrelli, L., Ploch, S., Shin, H.D., Shivas, R.G., Telle, S., Voglmayr, H., and Thines, M. 2015. *Towards a universal barcode of oomycetes – a comparison of cox1 and cox2 loci.* Mol Ecol Resour 15: 1275-1288.

Cooke, D., Drenth, A., Duncan, J.M. Wagels, G., and Brasier, C.M. 2000. *A molecular phylogeny of Phytophthora and related oomycetes.* Fungal Genet Biol. 30: 17-32.

Conners, I.L. 1967. *An annotated index of plant diseases in Canada and fungi recorded on plants in Alaska, Canada, and Greenland.* Res Bra Canada Dept Agri 1251: 1-381.

Constantinescu, O**.** 1991. *An annotated list of Peronospora names*. Thunbergia 15: 1-110.

Danielsen, S. 2001. *Heterothallism in Peronospora farinosa f.sp. chenopodii, the causal agent of downy mildew of quinoa (Chenopodium quinoa).* Journal of Basic Microbiology 41: 305-308.

Danielsen, S., Bonifacio, A., and Ames, T. 2003. *Diseases of quinoa (Chenopodium quinoa).* Food Reviews International 19: 43-59.

Danielsen, S., Mercado, V.H., Ames, T., and Munk, L. 2004. *Seed transmission of downy mildew (Peronospora farinosa f.sp. chenopodii) in quinoa and effect of relative humidity on seedling infection.* Seed Sci. & Technol. 32: 91-98.

Danielsen, S. and Ames, T. 2004. *Mildew (Peronsopora farinosa) of quinua (Chenopodium quinoa) in the Andean region.* International Potato Center.

Danielson, S., and Lubeck, M. 2010. *Universally primed-PCR indicates geographical variation of Peronospora farinose ex Chenopodium quinoa.* Journal of Basic Micrbiology 50: 104-109.

de Bary, A. 1863. *Recherches sur le developpement de quelques champignons parasites*. Ann. Sci. Naturelles. Botanique Ser.4 20:5-148.

Dekker, J. 1982. *Countermeasures for avoiding fungicide resistance*. In Fungicide resistance in crop protection, eds. Dekker, J and Georgopoulos, S. G. PUDOC, Wageningen, The Netherlands, 149-159.

Dick M.W. 1995. *Sexual reproduction in the Peronosporomycetes (chromistan fungi).* Can J Bot 73(Suppl. 1): S712-S724.

Domsch, K.H., Gams, W., Anderson, T.H. 1993. *Compendium of soil fungi* (Reprint [der Ausg. London] 1980. Ed.) Eching: IHW-Verl. ISBN 3980308383.

Ellis, Michael A. 2016. *Downy mildew of grape.* Ohio State University Extension. Accessed at https://ohioline.osu.edu/factsheet/plpath-fru-33 on April 2019.

Farr, D.F., & Rossman, A.Y. Fungal Databases, U.S. National Fungus Collections, ARS, USDA. Retrieved April 30, 2019, from https://nt.ars-grin.gov/fungaldatabases/

FAO. 2015. *Quinoa breaches the boundaries of outer space*. Retrieved on April 2019 from: http://www.fao.org/zhc/detail-events/en/c/283429/

Galwey, N.W. 1989. *Exploited plants – quinoa.* Biologist 36(5): 267-274.

Gaponenko, N.I. 1972. *The family Peronosporaceae of Central Asia and south Kazakhstan: a classification key*. (Translated from Russian). Publ. House Uzbek S.S.R., Tashkent, Fan, 344 pages.

Goker, M., Voglmayr, H., Riethmuller, A., and Oberwinkler, F**.** 2007. How do obligate parasites evolve? A multi-gene phylogenetic analysis of downy mildews. Fungal Genet. Biol. 44: 105-122.

Hudspeth, D.S.S., Nadler, S.A., and Hudspeth, M.E.S. 2000. *A COX2 molecular phylogeny of the Peronosporomycetes.* Mycologia 92: 674-684.

Hoang, D.T., Chernomore, O., von Haeseler, A., Minh, B.Q., and Vinh, L.S. 2018. *UFBoot2: Improving the ultrafast bootstrap approximation.* Mol Biol Evol 35: 518-522.

Ikeda, A. and Ichitani, T. 1985. *Density of Pythium zingiberum oospores in relation to infection of Chenopodium quinoa seedlings.* Bull. Univ. Osaka Pref. Ser. B 37: 13-18.

Inrinyi, L., Kovics, G.J., and Sandor, E. 2009. *Taxonomical re-evaluation of Phoma-like soybean pathogenic fungi*. Mycol. Res. 113: 249-260.

Irish, B.M., Correll, J.C., Feng, C., Bentley, T., and de los Reyes, B.G. 2008. *Characterization of a resistance locus (Pfs-1) to the spinach downy mildew pathogen (Peronospora farinosa f.sp. spinaciae) and development of a molecular marker linked to Pfs-1.* Phytopathology: 894-900.

Ishii, H. 2002. *Strobilurin resistance in cucumber pathogens: Persistence and molecular diagnosis of resistance.* In Modern fungicides and antifungal compounds III, eds. Dehne, H. W. et al., AgroConcept, Bonn, Germany, 149-159.

Ishii, H. 2006. *Impact of fungicide resistance in plant pathogens on crop disease control and agricultural environment.* JARQ 40(3): 205-2011.

Jackson, R.S. 2008. *Chapter 4: Vineyard practice.* In: Wine Science: Principles and Applications 3 pp. 143-206.

Jellen, E.N., Kolano, B.A., Sederber, M.C., Bonifacio, A., and Maughan., P.J. 2011. *Chenopodium in wild crop relatives: genomic and breeding resources: legume crops and forages.* Longdon, Springer Heidelberg Dordrect. 35-61.

Jing, L. Submitted 20-October-2015. GenBank Direct Submission. Xinjiang Agricultural University, Nanchang.

Kandel, S.L., Mou, B., Shishkoff, N., Shi, A., Subbarao, K.V., and Klosterman, S.J. 2019. *Spinach downy mildew: Advances in our understanding of the disease cycle and prospects for disease management.* Plant Disease 103: 791-803.

Katoh, K. and Standley, D.M. 2013. *MAFFT multiple sequence alignment software version 7: improvements in performance and usability.* Mol Biol Evol 30(4): 772-780.

Kitz, L. 2008. *Evaluation of downy mildew (Peronospora farinosa f. sp. chenopodii) resistance among quinoa genotypes and investigation of P. farinosa growth using scanning electron microscopy.* Thesis, Brigham Young University.

Kolano, B., Siwinska, D., and Maluszynska, J. 2008. *Comparative cytogenetic analysis of diploid and hexaploidy Chenopodium album.* Agg. Acta Soc Bot Pol 7: 293-298.

Kucmierz, J. 1966. *Parasitic fungi of the Ojcow National Park. Part II. Archimycetes, Phycomycetes, Ustilaginales*. Fragm. Florist. Geobot. 12: 497-511.

Kumar, A., Bhargava, A., Shukla, S., Singh, H.B., and Ohri, D. 2006. *Screening of exotic Chenopodium quinoa accessions for downy mildew resistance under mid-eastern conditions of India.* Crop Protection 25: 879-889.

Lapage, S.P., Sneath, P.H.A., Lessel., E.F., Skerman, V.B.D., Seeliger, H.P.R., and Clark, W.A. (1976 Revision) 1975. International Code of Nomenclature of Bacteria. American Society for Microbiology, Washington, DC. 180 pp.

Lizotte, E. 2015. *Managing hop downy mildew early in the season is critical.* Michigan State University Extension. Accessed at https://www.canr.msu.edu/news/managing_hop_downy_mildew_early_in_the_season_is_critical on April 2019.

Maughan, P.J., Chaney, L., Lightfoot, D.J., Cox, B.J., Tester, M., Jellen, E.N., and Jarvis, D.E. 2019. *Mitochondrial and chloroplast genomes provide insights into the evolutionary origins of quinoa (Chenopodium quinoa Willd.).* Nature Scientific Reports 9:185.

Mendes, M.A.S., da Silva, V.L., and Dianese, J.C. 1998. *Fungos em plants no Brasil.* Embrapa-SPI/Embrapa-Cenargen, Brasilia, 555 pages.

Mujica, A. 1994. *Andean grains and legumes.* In: Hernando Bernujo, JE., Leon, J (Eds.) Neglected Crops: 1492 from a Different Perspective, vol 26. FAO, Rome, Italy. Pp 131-148.

Muskett, A., and Malone, J. 1984. *Catalogue of Irish Fungi - V. Mastigomycotina and Zygomycotina*. Proc. Roy. Irish Acad., B. 84: 83-102.

Navruz-Varli, S. and Sanlier, N. 2016. *Nutritional and health benefits of quinoa (Chenopodium quinoa Willd.)* Journal of Cereal Science 69: 371-376.

Neff, E. 2017. *Developing a molecular pipeline to identify Chenopodium species in New England.* Thesis, University of New Hampshire.

Nemri, A., Atwell, S., Tarone, A.M., Huang, Y.S., Zhao, K., Studholme, D.J. Nordborg, M., Jones, J.D.G. 2010. *Genome-wide survey of Arabidopsis natural variation in downy mildew resistance using combined association and linkage mapping.* PNAS 107: 10302-10307.

Palomino, G., Hernandez, L.T., and de la Cruz Torres, E. 2008. *Nuclear genome size and chromosome analysis in Chenopodium quinoa and C. berlandieri subsp. nutalliae.* Euphytica 164: 221-230.

Phytophthora: habitat, symptoms, and reproduction. Accessed at http://www.biologydiscussion.com/fungi/phytophthora-habitat-symptoms-and-reproduction-mastigomycotina/23932 on April 2019.

Poland, J.A., Balint-Kurti, P.J., Wisser, R.J., Pratt, R.C., and Nelson, R.J. 2008. *Shades of gray: the world of quantitative disease resistance.* Trends in Plant Science 14:21-29.

Raja, H.A., Miller, A.N., Pearce, C.J., and Oberlies, N.H. 2017. *Fungal identification using molecular tools: a primer for the natural products research community.* J. Nat. Prod. 80: 756-770.

Risi, C.J. and Galwey, N.W. 1984. *The Chenopodium grains of the Andes: Inca crops for modern agriculture.* Adv. Appl. Biol. 10:145-216.

Riethmuller, A., Voglmayr, H., Goker, M. , Wiess, M., and Oberwinkler, F. 2002. *Phylogenetic relationships of the downy mildews (Peronosporales) and related groups based on nuclear large subunit ribosomal DNA sequences*. Mycologia 94: 834-849.

Sachay, D.J., Hudspeth, D.S.S., Nadler, S.N., and Hudspeth, M.E.S. 1993. *Oomycete mtDNA: Phytophthora genes for cytochrome c oxidase use an unmodified genetic code and encode proteins most similar to plants.* Exp Mycol 17: 7-23.

Schlick, G. and Bubenheim, D.L. 1996. *Quinoa – Candidate crop for NASA's controlled ecological life support systems.* In: Janick, J. (Ed.), Progress in New Crops. ASHS Press, Arlington, VA.

Shaner, G. and Finney, R.E. 1977. *The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat.* Phytopathology 67: 1051-1056.

Sleigh, M.A. 1990. *Protozoa and other protists.* Edward Arnold, London.

Stamatakis, A. 2014. *RAxML Version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies.* Bioinformatics 30: 1312-1313.

Testen, A.L., McKemy, M., and Backman, P.A. 2012. *First report of quinoa downy mildew caused by Peronospora variabilis in the United States.* The American Phytopathological Society 96: 146.

Testen, A.L. 2012. *Microbial approaches to support Andean quinoa production.* Thesis, The Pennsylvania State University.

Testen, A.L. and Backman, P.A. 2013. *First report of Ascochyta leaf spot of quinoa caused by Ascochyta sp. in the United States.* Plant Disease 97: 844.

Testen, A.L., del Mar Jimenez-Gasco, M., Ochoa, J.B., and Backman, P.A. 2014. *Molecular Detection of Peronospora variabilis in Quinoa Seed and Phylogeny of the Quinoa Downy Mildew Pathogen in South America and the United States.* The American Phytopathological Society 104: 379-386.

Tewari and Boyetchko. 1990. *Occurrence of Peronospora farinosa f. sp. Chenopodii on quinoa in Canada.* Canadian Plant Disease Survey 70: 127-128.

Thines, M., Voglmayr, H., and Goker, M. 2009. *Taxonomy and phylogeny of the downy mildews (Peronosporaceae).* In Oomycete Genetics and Genomics. Eds Lamour, K. and Kamoun, S. Wiley-Blackwell Publishing.

Thines, M. and Choi, Y.J. 2016. *Evolution, diversity, and taxonomy of the Peronosporaceae, with focus on the genus Peronspora.* Phytopathology Review 106: 6-18.

Torres, A.M., Weeden, N.F., and Martin A. 1993. *Linkage among isozyme, RFLP, and RAPD markers in Vicia faba.* Theor Appl Genet. 85: 937-945. Last revised 3 August 2005 by Keven deHaan.

USDA, 2015. United State Department of Agriculture. National Nutrient Database for Standard Reference Release, 28 (Basic Reports).

Walsh, B.M., Adhikary, D., Maughan, PJ., Emshwiller, E., and Jellen, E.N. 2015. *Chenopodium polyploidy inferences from Salt Overly Sensitive 1 (SOS1) data.* Am J Bot 102(4): 533-543.

Woudenberg, J.H.C., Hanse, B., van Leeuwen, G.C.M., Groenewald, J.Z., and Crous, P.W. 2017. *Stemphylium revisited.* Stud Mycol 87: 77-103.

Yerkes, W.D. and Shaw, C.G. 1959. *Taxonomy of the Peronospora species on Cruciferae and Chenopodiaceae*. Phytopathology 49:499-507.

Zhou, M. and Bai Q. Submitted 3-Aug-2017. GenBank Direct Submission. Agricultural College, Jilin Agricultural University.

APPENDICES

APPENDIX I: PREPARATION OF 2% CTAB BUFFER FOR DNA EXTRACTION

To make 100 ml:

In a large beaker on a stir plate mix:

M Tris-HCl (pH 8.0, 10 ml of 1 M stock)

20 mM Na-EDTA (4 ml of 0.5 M stock)

1.4 M NaCl (8.2 g)

2% (w/v) CTAB* (2.0 g)

*Hexadecyltrimethylammonium bromide (Sigma: H-5882)

Dissolve ingredients in sterile water, check pH and adjust to 8.0 if necessary.

APPENDIX II: QUANTIFICATION OF DNA USING A QUBIT FLUOROMETER

n = number of DNA samples + 2 standards

Working solution:

Reagent = 1 x n ul

Buffer = 199 x n ul

For each standard:

190 ul working solution + 10 ul standard

198 ul working solution + 2 ul DNA sample

The fluorometer is calibrated by analyzing Standard 1 and then Standard 2. Each DNA sample is then analyzed by the fluorometer, which then calculates the DNA concentration based on the volume of DNA solution added to each tube.

APPENDIX III: PREPARATION OF 1X TBE BUFFER FOR ELECTROPHORESIS GEL

To make 1 L of 10X TBE buffer:

In a large beaker on a stir plate, add 600 ml of sterile, filtered water. Add the following and stir until dissolved:

108 g Tris

55 g Boric acid

40 ml of 0.5 M NaEDTA

Adjust pH to 8.0, add enough sterile, filtered water to bring the volume to 1000 ml. Transfer the buffer to a large flask, cover the top with aluminum foil, and autoclave. Once cooled, dilute solution to 1X by adding 4500 ml sterile water to 500 ml 10X TBE. Store in a covered container.

APPENDIX IV: PYTHON SCRIPT ‘CLIPALIGNMENT.PY’ CODE TO TRIM SPECIFIC BASES OFF ALIGNMENTS

```
def parse_args():
    """ Parse arguments """
    parser = argparse.ArgumentParser()
    parser.add_argument("input", type=str, help="path to MSA")
    parser.add_argument("start", type=int, help="retained starting position")
    parser.add_argument("stop", type=int, help="retained stop position (exclusive)")

    return parser.parse_args()


def parse_fasta(input_path):
    """ Parse FASTA MSA file """
    entries = dict()

    # Open FASTA file
    active_header = ""
    with open(input_path, "r") as handle:
        for line in handle:
            line = line.rstrip()

            # Check for header or sequence
            if line.startswith(">"):
                active_header = line[1:]
                entries[active_header] = ""
            else:
                entries[active_header] += line
```

```
    return entries



args = parse_args()
entries = parse_fasta(args.input)

# Clip entries and print
for entry in entries:
    print(">{}\n{}".format(entry, entries[entry][args.start:args.stop]))

## usage: clip_alignment.py [-h] input start stop
# ,./clip_alignment.py /path/to/your/file.ali 40 65 > newfile.ali

# positional arguments:
  #input       path to MSA
  #start       retained starting position
  #stop        retained stop position (exclusive)

#optional arguments:
  #-h, --help  show this help message and exit
```